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Influence of Thyrotrophin on Growth of Transplantable Thyroid-Gland Tumors of Mice [1]

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Summary

The administration of diets high or low in iodide did not affect the growth rate of independent, mouse, transplantable thyroid-gland tumors of sublines 3C and 4JW. The inclusion of propylthiouracil (PTU) in the diet increased the growth rate at the 6th week and resulted in an alteration in the morphology of the tumors of subline 3C. No significant change could be detected in the histologic features or tumor growth of the 4JW and 6 tumor sublines in the PTU-treated animals. Exogenous thyrotrophin administered for 33 days to mice bearing lines 3C or 4JW resulted in some increased growth of line 3C tumors and some slowing of growth of line 4JW tumors. Similar effects on tumor growth of these lines were obtained in mice subtotally thyroidectomized with radioiodide. In no case was the activity of the iodide pump significantly altered. The thyroid glands of tumor-bearing mice fed PTU did not enlarge as rapidly beyond 3 weeks as did the glands of non-tumor-bearing mice ingesting the goitrogen.—**J. Nat. Cancer Inst. 22: 443–461, 1959.**

During the first phases of the development of experimental mouse thyroid-gland tumors, increased amounts of circulating thyrotrophin (TSH) must be maintained in the animals for prolonged periods. The removal of goitrogen from the diet during the early periods resulted in growth failure of the thyroid-tumor tissue (*1*).

Most of several lines of thyroid-gland tumors of the mouse, however, eventually became independent, *i.e.*, able to continue growth without more than the amounts of TSH elaborated by an animal on a goitrogen-free diet. Morris (*2*) reported increased growth rate of dependent thyroid-gland tumors of one subline growing in hosts ingesting a goitrogen-containing diet, but the growth of independent thyroid-gland tumors of another subline was unaffected by goitrogen ingestion, Wollman *et al.* (*3*). Furthermore, Wollman *et al.* (*4*) reported that the ability of subline 3C tumors to trap iodide was unaffected by goitrogen ingestion. Such obser-

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vations seem to indicate considerable variation in the effect of TSH on growth and on utilization of the TSH by mouse thyroid-gland tumors.

The separation of the effect of TSH on thyroid-gland function into that of growth and of hormone synthesis has assisted in a further analysis of the influence of TSH on thyroid-gland neoplasms. One criterion of hormone synthesis is the accumulation of iodide by the thyroid gland. This function can be evaluated by the ratio of the radioiodide concentration in a goitrogen-blocked gland to the radioiodide in a certain volume of serum (T/S) (*5*).

The experiments reported herein were designed to study the utilization of endogenous and exogenous TSH in thyroid-tumor-bearing mice. The parameters of function studied were 1) the thyroid-gland growth response to ingested goitrogen in thyroid-tumor-bearing host mice, and 2) the effect of exogenous TSH and endogenously induced secretion of TSH (by subtotal thyroidectomy) and by goitrogen ingestion on thyroid-tumor growth. The data indicate that increased TSH secretion in goitrogen-treated, tumor-bearing mice resulted in histologic changes in one tumor line with no effect on iodide pump activity. Furthermore, it was noted that the thyroid glands of tumor-bearing mice fed goitrogen failed to grow at the same rate as thyroid glands in non-tumor-bearing animals.

Methods

Weanling C3H/HeN male mice, used in all experiments described in this paper, were adapted to the NCI pellet diet over a period of 1 month, and then placed on the basal low-iodine diet #77,[5] or modifications of this diet, as follows:

a) Diet #265 = #77 + 0.025 percent propylthiouracil (PTU).

b) Diet #267 = #77 + added KI equivalent to 2.1 μg. of iodine per gm. of diet.[6]

c) Diet #268 = #267 + 0.025 percent PTU.

Mice ranging in age from 2 to 3 months were inoculated with weighed pieces of thyroid-tumor tissue obtained from a single animal, or at most 2 animals, bearing either tumor subline 3C, 4JW, or 6. If tumors from 2 animals were used, the recipients were marked and later checked for differences in tumor behavior; the differences were so slight they were considered of negligible importance in these experiments. The mice were housed in raised, wire, cloth-bottom cages, 5 animals per group. Food and water were available at all times.

The activity of the thyroid iodide pump (T/S) was determined by the method of Halmi *et al.* (*5*). A single blocking dose of 1 mg. of PTU, administered subcutaneously (*6*), was followed 45 minutes later by a

[5] Diet 77 for these experiments contained the following ingredients: Skim-milk powder, 22.75%; whole, hard, spring-wheat meal, 61.52%, dry debittered Brewer's yeast, 4.00%; U.S.P. NaCl (Mallinkrodt), 1.40%; ferric citrate, 0.13%; cod-liver oil, 0.50%; and corn oil, 9.70%. The cod-liver-oil content of this version of diet 77 was reduced from 2.00 to 0.5%, and the corn-oil content was increased from 8.20 to 9.70%. Diet 77 contained, according to our analyses, 180 ± 25 μg. iodine per kg.

[6] A solution of KI in acetone was thoroughly mixed with skim-milk powder. The solvent was evaporated and a portion of the resulting iodized milk powder added to untreated milk powder to give a diet containing the requisite amount of iodine.

1 μc subcutaneous injection of I^{131}.[7] The mice were killed, by ether anesthesia, 60 minutes after I^{131} administration and withdrawal of 0.5 to 1.0 ml. of blood from the abdominal aorta. The thyroid glands and tumors were immediately dissected at 9× magnification and weighed on a Roller-Smith torsion balance. The T/S ratio of both thyroid gland and tumor was determined from the ratio of radioiodide per 100 mg. of tissue to radioiodide per 0.1 ml. of serum. The standard errors were calculated by the short method of Mantel (7).

Preliminary tests showed that acute doses of PTU, ranging from 0.5 mg. to 3.0 mg., were equally effective in blocking the uptake of I^{131} (table 1) by the mouse thyroid gland. This acute blocking effect of

TABLE 1.—*Effect of graded acute doses of PTU on completeness of blocking (5 mice per group)*

Body weight (*gm.*)	Thyroid weight (*mg.*)	PTU (*mg.*)	T/S
25.5 ± 0.5	1.6 ± .06	0.0	1773 ± 331
25.4 ± 2.0	1.6 ± .14	0.5	227 ± 19
24.2 ± 1.3	1.6 ± .12	1.0	259 ± 11
23.2 ± 1.5	1.4 ± .16	2.0	319 ± 26
24.4 ± 1.4	1.4 ± .18	3.0	250 ± 32

TABLE 2.—*Duration of blocking effect of acute dose of 1.0 mg. PTU per mouse (5 mice per group)*

Time between PTU blocking and I^{131} administration (*hours*)	Body weight (*gm.*)	Thyroid weight (1 lobe) (*mg.*)	T/S
0.5	24.8 ± .6	.94 ± .11	116 ± 11
1.0	23.4 ± .8	.87 ± .10	132 ± 11
2.0	23.6 ± .8	.80 ± .08	123 ± 15
3.0	23.6 ± .4	.82 ± .08	126 ± 17
4.0	22.2 ± .4	.87 ± .07	149 ± 8

PTU on I^{131} uptake lasted for at least 4 hours (table 2). The effectiveness of the blocking dose in the animals with goitrous glands was not tested, but since these animals had been consuming a diet containing goitrogen it was assumed that the acutely administered PTU supplemented existing levels of goitrogen in the body fluids.

For experiments involving exogenous TSH, a solution of thyrotrophin [8] was prepared which contained 1.0 mg. equivalent of the U.S.P. Reference Substance per ml. Aliquots of this solution sufficient for a day's injections were frozen in separate vials and stored at subzero temperature until used. Subcutaneous daily injections of the TSH solution from 1 vial, warmed to room temperature, were made for a period of 33 days. Each mouse

[7] Obtained from the Oak Ridge National Laboratories, Oak Ridge, Tennessee, on allocation from the Atomic Energy Commission.

[8] Supplied by Armour Laboratories, Chicago, Ill., through the courtesy of Dr. I. Bunding. Each vial, Lot # 377157, contained the equivalent of 40 mg. U.S.P. Reference Substance.

received 0.1 mg. TSH per day divided into 2 equal doses. Any excess remaining after each day's injections was discarded.

In experiment I, mice were placed on the 4 diets immediately after inoculation with tumor 3C. Five animals from each group were killed at 3-week intervals, for a total of 12 weeks. Body weights, food intakes, and weights and the T/S ratios of the tumors and the thyroid glands were determined. The experiment was repeated on non-tumor-bearing mice and on mice inoculated with tumor lines 4JW and 6.

In experiment II, the direct effect of exogenous and endogenous thyrotrophin on tumor growth was studied. Twenty-eight mice were inoculated with tumor subline 3C. Seven mice served as controls and 7 mice received the equivalent of 0.1 mg. U.S.P. Thyrotrophin Reference Substance daily for 33 days. The other 14 mice were partially thyroidectomized by subcutaneous injection of 30 μc I^{131} 2 weeks prior to tumor inoculation, for the purpose of augmenting elaboration of endogenous TSH. Seven of these mice received 0.1 mg. of the TSH preparation daily for 33 days, and the remaining 7 served as subtotally thyroidectomized controls. The experiment was duplicated by use of another tumor subline, 4JW.

Three experimental approaches were used to increase the level of circulating TSH: 1) Endogenous TSH secretion was induced by the administration of PTU. 2) Exogenous TSH was administered. 3) This approach, properly called excitation of endogenous secretion but included as a part of the second experiment, was the attempted stimulation of TSH production by subtotal thyroidectomy. The latter procedure was used in the hope of disclosing any antihormone effects that might follow prolonged administration of exogenous TSH.

Results

Experiment I.—Effect of Endogenous Thyrotrophin on Thyroid-Gland Growth in Tumor-Bearing Mice

The 3C thyroid-gland tumor grew at a greater rate in the presence of PTU (significant at the 5% level) than did the tumor in mice fed a goitrogen-free diet (table 3). This effect appeared at the 6-week interval and disappeared by 12 weeks. Iodide pump activity of the tumors remained uninfluenced by the dietary regimen (table 3). Added iodide (diet #267) depressed the T/S of the thyroid gland and PTU + iodide (#268) augmented the T/S in the glands of these tumor-bearing mice.

The influence of PTU ingestion was studied in two other lines of thyroid tumors. No effect of PTU ingestion on tumor weights or tumor T/S was found for either tumor line 4JW or 6 at the intervals studied (table 4). The T/S of the thyroid glands of non-tumor-bearing mice ingesting PTU for 6 or more weeks, however, was depressed (table 4). The T/S of the thyroid glands of 4JW tumor-bearing mice was also depressed, but the T/S of the thyroid glands of line 6 (table 4) and line 3C (table 3) tumor-bearing mice was not significantly lower than the T/S values ob-

TABLE 3.—*Mean weights and T/S ratios of thyroid glands and thyroid-gland tumors (subline 3C) in groups of animals receiving 4 different diets*

Weeks after inoculation	Tumor weight (*mg.*)	Thyroid weight (*mg.*)	T/S tumor	T/S thyroid
		Diet #77		
3	3.9 ± 0.8*	1.3 ± 0.1	.95 ± 0.2	281.6 ± 16.0
6	12.7 ± 3.0	1.6 ± 0.2	.52 ± 0.2	136.6 ± 18.7
9†	156.6 ± 64.5	1.8 ± 0.3	.64 ± 0.2	199.4 ± 68.5
12	289.0 ± 133.5	1.8 ± 1.3	.42 ± 0.03	146.4 ± 20.0
		Diet #265		
3	6.4 ± 1.4	14.2 ± 0.8	.73 ± 0.2	144.6 ± 13.0
6	111.5 ± 56.8	18.5 ± 3.8	.72 ± 0.3	132.0 ± 12.6
9	182.9 ± 75.7	19.4 ± 2.3	.56 ± 0.1	145.4 ± 12.7
12	219.4 ± 76.0	25.5 ± 4.6	.42 ± 0.04	152.1 ± 14.9
		Diet #267		
3†	4.9 ± 1.2	2.2 ± 0.2	.37 ± 0.1	50.5 ± 7.3
6	44.6 ± 18.5	1.6 ± 0.2	.50 ± 0.1	84.4 ± 12.8
9	137.6 ± 46.8	2.3 ± 0.1	.52 ± 0.02	59.8 ± 9.7
12	368.1 ± 120.3	1.9 ± 0.1	.42 ± 0.1	44.9 ± 16.7
		Diet #268		
3	7.0 ± 2.5	5.2 ± 0.7	.70 ± 0.2	159.2 ± 12.9
6	88.7 ± 28.7	9.9 ± 2.6	.56 ± 0.03	179.8 ± 24.2
9†	228.2 ± 60.9	7.1 ± 1.3	.59 ± 0.2	308.1 ± 65.0
12†	472.2 ± 117.7	6.6 ± 0.6	.56 ± 0.1	255.6 ± 27.8

*Mean and standard error of the mean (S.E.).

$$\text{S.E.} = \frac{\text{difference between highest and lowest values}}{\text{number of animals}} \quad (7).$$

†Four mice in all †groups; 5 mice in all other groups.

tained for their tumor-bearing controls on diet #77 or their non-tumor-bearing controls ingesting PTU (table 4).

The influence of transplantable thyroid-gland tumors upon the response of the hosts' thyroid glands to the goitrogen PTU was studied in groups of mice bearing three different thyroid-tumor sublines, namely, 3C, 4JW, and 6. The averaged results in groups of animals, each comprised of 4 or 5 mice, are presented in text-figure 1. The rate of thyroid hypertrophy in PTU-treated mice with tumors declined as the tumors increased in size. Compare curve A, the weight of thyroid glands of non-tumor-bearing (control) mice, with the weights of the thyroid glands of tumor-bearing mice (curves B, C, and D). The thyroid glands of subline 3C tumor-bearing mice grew at approximately the same rate for the first 6 weeks but at a slower rate after 6 weeks than those of non-tumor-bearing mice (curve B compared to curve A). The thyroid glands of mice bearing thyroid-gland tumors of sublines 4JW (curve C) and 6 (curve D)

TABLE 4.—*Mean weights and T/S ratios of thyroid glands and thyroid-gland tumors of mice bearing two tumor sublines, and of thyroid glands of non-tumor-bearing mice on diets with and without PTU*

Diet #	Weeks on experiment	Tumor weight (*mg.*)	Thyroid weight (*mg.*)	T/S tumor	T/S thyroid
No tumor					
77	3		2.0 ± 0.2*		97 ± 9
	6		1.7 ± 0.1		170 ± 18
	9		1.7 ± 0.1		172 ± 9
	12		1.8 ± 0.1		198 ± 22
265	3		14.9 ± 0.9		137 ± 5
	6		19.3 ± 2.3		123 ± 6
	9		29.1 ± 2.7		106 ± 10
	12		37.9 ± 5.9		87 ± 9
Tumor subline 4JW					
77	10	238 ± 40	1.9 ± 0.1	.44 ± .02	220 ± 42
	13	574 ± 230	1.6 ± 0.3	.42 ± .03	208 ± 15
265	10	272 ± 98	17.3 ± 0.9	.44 ± .05	109 ± 13
	13	604 ± 156	19.7 ± 4.2	.40 ± .05	108 ± 10
Tumor subline 6					
77	4	16 ± 8	1.56 ± 0.1	.56 ± .15	129 ± 14
	7	374 ± 140	1.88 ± 0.2	.42 ± .01	98 ± 17
	10	982 ± 275	1.82 ± 0.2	.50 ± .03	129 ± 29
265	4	48 ± 18	13.1 ± 1.8	.54 ± .08	147 ± 10
	7	464 ± 160	16.1 ± 1.0	.54 ± .05	59 ± 3
	10	1205 ± 191	15.0 ± 2.6	.56 ± .04	190 ± 16

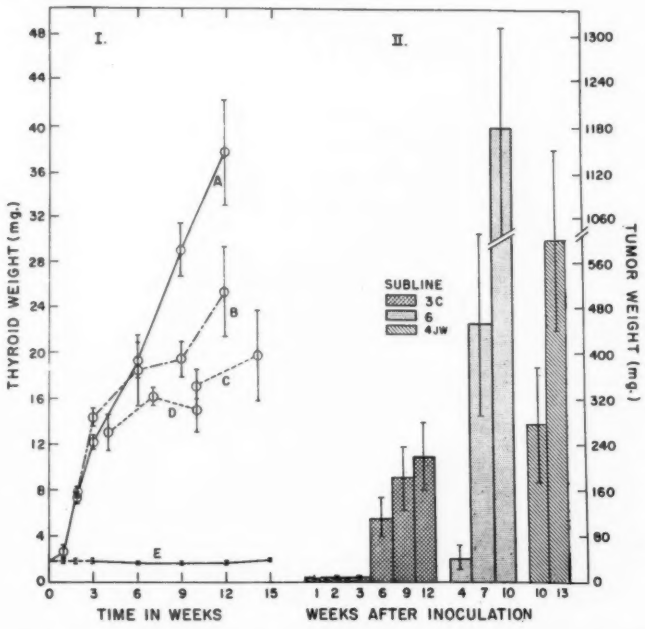
*S.E. calculated as in table 3.

showed no significant enlargement during 4 to 10 and 10 to 13 weeks respectively—periods during which rapid tumor growth occurred. The thyroid weights of these latter two groups of tumor-bearing mice were not obtained for earlier periods. The thyroid glands of tumor-bearing and non-tumor-bearing mice showed approximately the same response to the ingestion of PTU during the first 4 to 6 weeks of the experiment, especially in mice bearing tumor sublines 3C and 6. It was after 6 weeks that the most rapid tumor growth took place. Curve E shows the weight of normal (control) thyroid glands of mice not receiving PTU.

As shown in table 5, the increase in weight of the non-tumor-bearing control mice, at 9- and 12-week intervals, was more than for any other group. The increase in weight of the tumor-bearing control mice was also somewhat greater than for either tumor-bearing or non-tumor-bearing mice ingesting PTU. At the intervals studied there was no significant difference in terminal body weights between tumor-bearing and non-tumor-bearing mice on the PTU diet (table 5). Their weights either did not change or decreased during the periods studied except for the tumor-

bearing groups of line 6 which had some increase in weight. The food intake of the non-tumor and the tumor-bearing control mice (table 5) was slightly higher than for either tumor-bearing or non-tumor-bearing mice ingesting PTU, except for 4JW tumor-bearing mice. The food intake of PTU-fed mice bearing tumor 3C was less than for the other groups. These animals also lost the most weight.

Figure 1 shows a representative section of the thyroid-tumor tissue of subline 3C, from the sixteenth transplant generation, used as a source of tumor to inoculate the mice in this experiment. The changes during successive periods of 3, 6, 9, and 12 weeks of ingestion of diet #265 (PTU-containing diet) on the histologic features of the tumor is illustrated in figures 2, 3, 4, and 5, respectively. Noteworthy is the progressive decrease in the number and size of the follicles. Almost none remain after 12 weeks. These histologic features of tumor line 3C appear to be influenced by PTU feeding up to 12 weeks, presumably through increased pituitary secretion of TSH. The T/S ratio of the tumor, however, is insensitive to the goitrogen, a finding originally noted by Wollman *et al.* (*4*) and reconfirmed by the data presented in the present report.



TEXT-FIGURE 1.—I. Average weight of thyroid glands of mice with and without tumors. A) PTU non-tumor-bearing control. B, C, and D) Mice receiving PTU and bearing tumor sublines 3C, 4JW, and 6, respectively. E) Non-tumor-bearing controls without PTU.

II. Average weight of thyroid-gland tumors of groups listed above carrying tumor sublines 3C, 6, and 4JW at various intervals from 1 to 13 weeks after inoculation. The *vertical* lines designate the standard error of the mean.

TABLE 5.—*Average body weight and average daily food intake of non-tumor-bearing and tumor-bearing mice*

Tumor subline	Duration of experiment (*weeks*)	Average body weight			Average daily food intake per mouse (*gm.*)
		Initial (*gm.*)	Terminal (*gm.*)	Change in weight (*gm.*)	
Non-tumor-bearing, diet #77 (no PTU)					
0	3	27.2*	27.2 ± 0.6†	0	3.2 ± .07
	6	24.0	25.4 ± 1.0	1.4	2.8 ± .17
	9	21.6	28.0 ± 2.0	6.4	2.9 ± .10
	12	25.0	35.2 ± 2.8	10.2	3.2 ± .06
Tumor-bearing, diet #77 (no PTU)					
3C	3	22.4 ± 1.5	24.4 ± 1.7	2.0	2.8 ± .07
	6	23.4 ± 1.8	27.9 ± 0.4	4.5	2.8 ± .05
	9	21.2 ± 1.4	26.4 ± 3.5	5.2	2.8 ± .08
	12	23.3 ± 1.4	26.0 ± 3.6	2.7	2.5 ± .05
4JW	10	23.6	26.8 ± 1.2	3.2	3.1 ± .09
	13	22.8	26.0 ± 0.8	3.2	3.0 ± .08
6	4	21.5 ± 0.7	23.7 ± 1.2	2.2	2.8 ± .04
	7	22.2 ± 1.7	25.6 ± 1.4	3.4	2.9 ± .04
	10	23.7 ± 1.4	24.7 ± 1.8	1.0	2.7 ± .03
Non-tumor-bearing, diet #265 (PTU)					
0	3	22.8	24.6 ± 1.0	1.8	2.7 ± .10
	6	22.8	23.0 ± 0.6	0.2	2.8 ± .17
	9	24.0	24.4 ± 1.2	0.4	2.7 ± .11
	12	25.2	22.2 ± 1.4	−3.0	2.7 ± .09
Tumor-bearing, diet #265 (PTU)					
3C	3	23.3 ± 0.7	23.3 ± 0.7	0	2.2 ± .06
	6	22.5 ± 1.1	21.3 ± 1.2	−1.2	2.2 ± .04
	9	23.5 ± 0.6	22.0 ± 1.5	−1.5	2.1 ± .03
	12	23.4 ± 1.4	20.6 ± 1.6	−2.8	2.0 ± .04
4JW	10	25.2	24.4 ± 1.0	−0.8	3.0 ± .11
	13	23.0	23.2 ± 2.2	0.2	2.9 ± .07
6	4	21.2 ± 1.3	23.7 ± 1.2	2.5	2.6 ± .04
	7	21.7 ± 0.7	24.8 ± 0.6	3.1	2.6 ± .02
	10	22.7 ± 0.6	24.3 ± 1.4	1.6	2.6 ± .03

*Where there are no standard errors given, the animals were weighed as a group.
†Standard errors calculated as in table 3.

A section of the donor tumor of subline 4JW, fifteenth transplant generation, is presented in figure 6. The histologic characteristics of tumors of this subline growing in mice on diet #265 after 10 and 13 weeks, respectively, are shown in figures 7 and 8.

Variable histologic patterns of tumor subline 6 (fifteenth transplant generation) are evident in figures 9 and 10. Microscopic features of the sixteenth generation of this tumor line growing in mice on diet #265 for

4 and 12 weeks are shown in figures 11 and 12, respectively. In contrast with subline 3C tumors, PTU appeared to have slight if any effect on the histology of tumors of sublines 4JW and 6.

The sixteenth transplant generation of subline 3C resembles the tenth transplant generation of this tumor line (*3*). Tumors of line 4JW are descendents of line 4HJK (*3*), and had been growing as independent tumors since the eleventh transplant generation. Morphologic features of the fifteenth transplant generation of independent subline 4JW tumor differ from those of the tenth-generation dependent tumor subline 4HJK (*3*, *4*) in having smaller follicles and in appearing as a more solid-cell type of tumor. Tumor line 6, on the other hand, shows a more variable histologic character than the other two tumor lines (figs. 9 and 10) and contrasts considerably from the tenth-generation line 6 tumor (*3*). Growth depression of the thyroid glands of tumor-bearing hosts fed PTU took place independently of alteration in tumor morphology (text-fig. 1).

Experiment II.—Effect of Exogenous Thyrotrophin and Subtotal Thyroidectomy on Growth of Thyroid-Gland Tumors

The histologic pattern of the thyroid gland of a normal animal, as used in this experiment, is illustrated in figure 13. The numerous, uniformly distributed follicles are surrounded by slightly elevated epithelial cells. Evidence of successful partial thyroidectomy can be noted in figures 14 and 15, in which extensive disorganization of the follicular structure and large amounts of connective tissue are observed. Very few follicles are present. There seem to be somewhat more extensive changes in the thyroid gland in the group carrying line 3C tumor, as represented in figure 15, than in the non-tumor-bearing animals (fig. 14). If there were an effect from treating mice with exogenous TSH it could not be recognized by changes in thyroid-gland histology (*cf.* fig. 16 with fig. 13).

That a subtotal thyroidectomy was obtained is also indicated by the heavier weights of the pituitary glands of the I^{131}-treated animals. It will be noted that injections of exogenous TSH into subtotally thyroidectomized mice, twice daily, failed to influence pituitary weight (table 6). The average size of subline 3C tumors was doubled during TSH treatment,

TABLE 6.—*Pituitary weight*

Subline	Number of mice	Treatment	
		No TSH (*mg.*)	TSH (*mg.*)
3C	7	1.50 ± .09	1.42 ± .09
4JW	7	1.53 ± .02	1.51 ± .09
Subtotal thyroidectomy			
3C	7	2.29 ± .14	2.20 ± .24
4JW	5	2.61 ± .07	
4JW	7		2.44 ± .05

while the average size of the subline 4JW tumors seemed depressed by TSH though the degree of significance was low in both experiments (table 7). The ability of the two tumor sublines to pump iodide was unaffected by exogenous TSH (table 8), though the T/S and weight of the thyroid gland in subtotally thyroidectomized mice was depressed. This is a further indication of the partial destruction of the thyroid.

The administration of TSH to partially thyroidectomized tumor-bearing mice of sublines 3C and 4JW resulted in the same tumor response to TSH as occurred in nonthyroidectomized animals. Any additional elaboration of TSH by the pituitary gland in the partially thyroidectomized mice produced no recognizable effect in either tumor subline over that caused by the exogenous TSH administered.

Discussion

Thyrotrophin of both endogenous and exogenous origin exerted different and unpredictable effects on the experimental thyroid-gland tumors. The hormone had no effect on the iodide pump activity of those tumors. In the presence of augmented blood titers of TSH, subline 4JW grew at a slower rate than when grown in mice having control-blood TSH levels. This slower growth seems unlikely to be attributed to elevated metabolic activity of the mice since the thyroid glands of animals receiving exogenous TSH were not enlarged nor did they show increased pump activity. In the radioiodide-subtotally-thyroidectomized mice the pituitary glands were enlarged, yet the thyroid glands did not respond to the augmented TSH blood titers. The lack of apparent effect of the exogenous TSH on the thyroid glands of the recipient mice may be due to the small amount of hormone administered or to the formation of antihormones. The latter suggestion seems unlikely because some tumor weights were affected.

Judging by tumor weights, one of 2 tumor sublines studied (4JW) failed to be stimulated but rather was depressed by exogenous TSH, while the other, subline 3C, showed to a low degree a significantly increased growth response. Tumor growth rates of sublines 6 and 4JW were unaffected by PTU as compared with the tumor growth rate in mice fed the goitrogen-free diet. Thus, little if any effect could be found for either exogenous or endogenous TSH on the growth rate of subline 4JW or for endogenous TSH on subline 6.

The administration of PTU to mice and the subsequent elevation of the blood levels of TSH result in thyroid-gland enlargement in tumor-bearing mice. However, these glands do not grow as rapidly as do the thyroid glands of non-tumor-bearing mice fed diets containing comparable amounts of PTU and iodide. Wollman *et al.* (*8*) also noted in a single experiment a partial trend toward normal thyroid weight in thyroid-gland tumor-bearing mice. Their observations, however, were not designed to specifically characterize such an effect and the animals were taken off the goitrogen 2 weeks before the experiments were terminated.

The subline 3C tumor, although an autonomous tumor, still clearly showed a morphologic response to the high levels of TSH induced in the

TABLE 7.—*Effect of exogenous thyrotrophin (TSH) on average weight of thyroid-gland tumors in intact and subtotally thyroidectomized mice*

Tumor subline	TSH			No TSH		
	Number of mice	Procedure	Tumor weight*	Number of mice	Procedure	Tumor weight
3C	7	Intact	$20.15 \times \div 1.27$	7	Intact	$8.72 \times \div 1.31$
3C	7	S.T.T$_x$.†	$13.39 \times \div 1.27$	7	S.T.T$_x$.	$8.84 \times \div 1.22$
Combined 3C	14		$18.48 \times \div 1.16$	14		$8.78 \times \div 1.13$
4JW	7	Intact	$7.99 \times \div 1.34$	7	Intact	$15.16 \times \div 1.18$
4JW	8	S.T.T$_x$.	$12.74 \times \div 1.20$	6	S.T.T$_x$.	$19.51 \times \div 1.60$
Combined 4JW	15		$10.25 \times \div 1.14$	13		$17.03 \times \div 1.24$

*Tumor weights in this table were derived from the logarithmic means. Converting the data in this table to logarithms tends to correct for skewness which cannot be accomplished by the usual statistical methods based on normal distributions around arithmetic means. Making the analyses from logarithmic transformation of the data also minimizes the effects of widely divergent findings. The standard error for logarithmic means is expressed as a multiplier (or divisor) rather than as ±, *e.g.*, one S.E. limit for the combined 3C tumors receiving TSH is $\frac{18.48}{1.16}$ = 15.93 or $18.45 \times 1.16 = 21.44$ and 3C tumors without TSH is $\frac{8.78}{1.13} = 7.78$ or $8.78 \times 1.13 = 9.92$ while 2 S.E. limits for TSH-treated mice would be $\frac{18.48}{(1.16)^2} = 13.69$ or $18.48 \times (1.16)^2 = 24.95$ and 2 S.E. limits for non-TSH-treated mice would be $\frac{8.78}{(1.13)^2} = 6.96$ or $8.78\ (1.13)^2 = 11.24$. These limits are not symmetrical, cannot go below 0, and go up faster than they go down.

†S.T.T$_x$. = subtotally thyroidectomized.

TABLE 8.—*Effect of exogenous thyrotrophin and subtotal thyroidectomy on growth of the thyroid gland and the T/S ratios of tumor and thyroid gland*

A

Subline	Tumor					
	TSH			No TSH		
	Number of mice	Tumor weight* (*mg.*)	T/S	Number of mice	Tumor weight (*mg.*)	T/S
3C	7	23.7 ± 6.7	.33 ± .03	7	10.6 ± 2.1	.54 ± .11
4JW	7	10.4 ± 3.1	.54 ± .07	7	19.4 ± 5.4	.57 ± .16
Tumor—after subtotal thyroidectomy						
3C	7	20.3 ± 4.8	.43 ± .62	7	10.0 ± 2.0	.44 ± .03
4JW	7	14.2 ± 2.5	.59 ± .05	5	30.1 ± 11.9	.54 ± .03

B

Subline	Thyroid gland					
	Number of mice	Thyroid weight (*mg.*)	T/S	Number of mice	Thyroid weight (*mg.*)	T/S
3C	7	1.96 ± .06	172 ± 16.3	7	1.97 ± .09	180 ± 9.6
4JW	7	2.13 ± .17	216 ± 17.2	7	2.09 ± .12	173 ± 18.2
Thyroid—after subtotal thyroidectomy						
3C	7	.81 ± .09	97 ± 12.4	7	.80 ± .15	60 ± 15.0
4JW	7	.38 ± .06	39 ± 7.4	5	.54 ± .13	49 ± 7.4

*Tumor weights in this table are arithmetic means.

PTU-fed mice. The effect of increased TSH due to the ingestion of PTU on the histology of the other tumor lines, 6 and 4JW, was not recognizable.

Crile (*9*) has reported that some thyroid cancers in man were made to regress or disappear by treating the patient daily with desiccated thyroid. His theory was that the desiccated thyroid material suppressed the elaboration of TSH by the pituitary gland. This concept is the reverse of that tried in these experiments where exogenous TSH was administered or where subtotal thyroidectomy was performed to increase the secretion of endogenous TSH.

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*Tumor weights in this table are arithmetic means.

PLATES

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PLATE 38

FIGURE 1.—A section of thyroid-tumor line 3C, sixteenth transplant generation. The host carrying this tumor was on diet #77. Hematoxylin and eosin. × 27

FIGURE 2.—Line 3C tumor in an animal ingesting diet #265 for 3 weeks. Gomori-trichrome. × 270

FIGURE 3.—Line 3C tumor in a mouse ingesting diet #265 for 6 weeks. Gomori-trichrome. × 270

FIGURE 4.—Line 3C tumor in a mouse ingesting diet #265 for 9 weeks. Gomori-trichrome. × 270

FIGURE 5.—Line 3C tumor in a mouse ingesting diet #265 for 12 weeks. Gomori-trichrome. × 270

FIGURE 6.—A section of thyroid tumor line 4JW, fifteenth transplant generation. The host bearing this tumor received diet #77. Hematoxylin and eosin. × 270

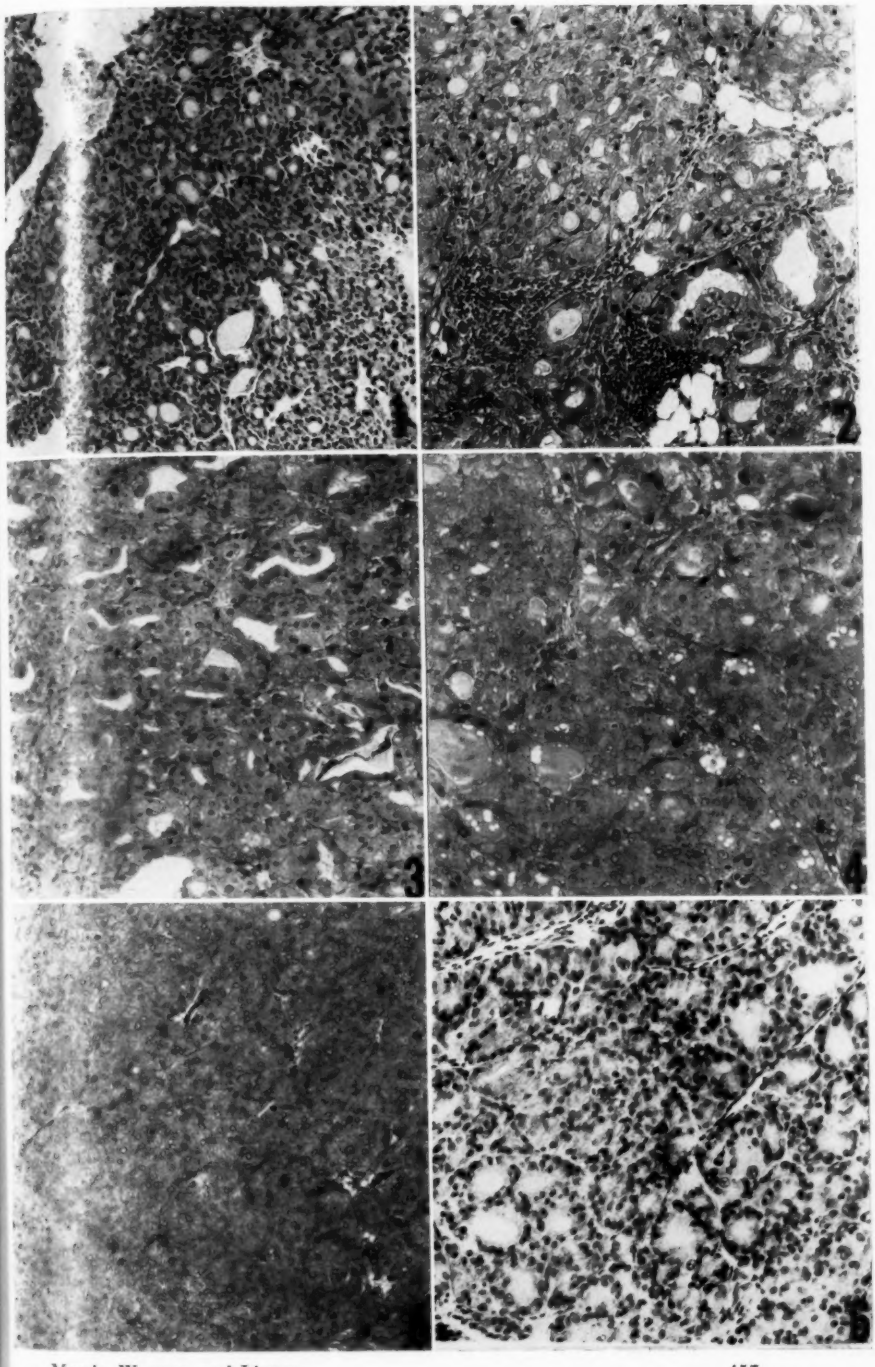

PLATE 39

FIGURES 7 and 8.—Thyroid-tumor line 4JW after 10 and 13 weeks, respectively, on diet #265. Comori trichrome. × 270

FIGURES 9 and 10.—Sections of thyroid-tumor line 6, fifteenth generation, from two different hosts. Both hosts carrying this tumor ingested NCI pellets. Hematoxylin and eosin. × 270

FIGURES 11 and 12.—Sections of tumors (line 6) after 4, and figure 12 after 7 weeks on diet #265, respectively. Gomori-trichrome. × 270

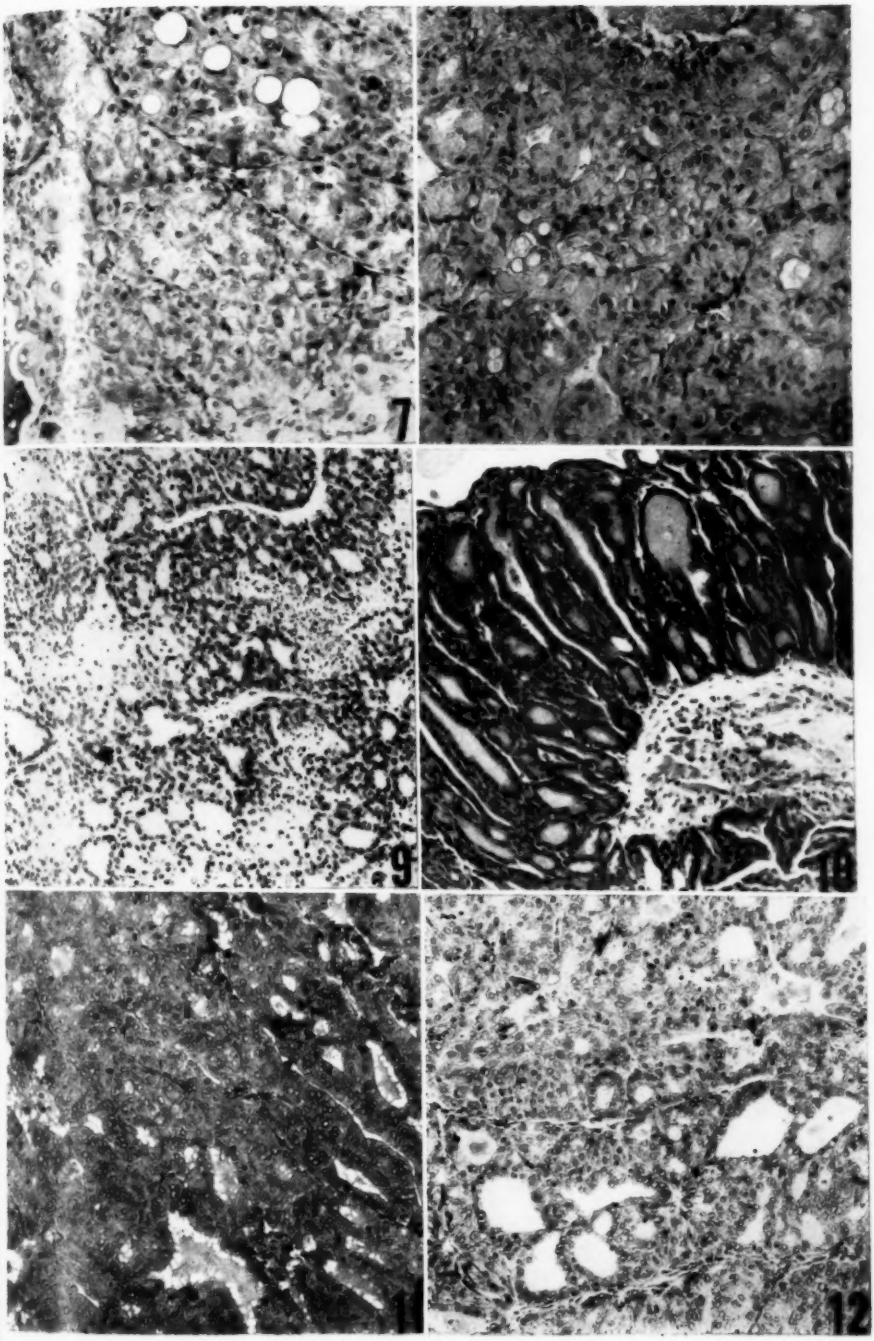
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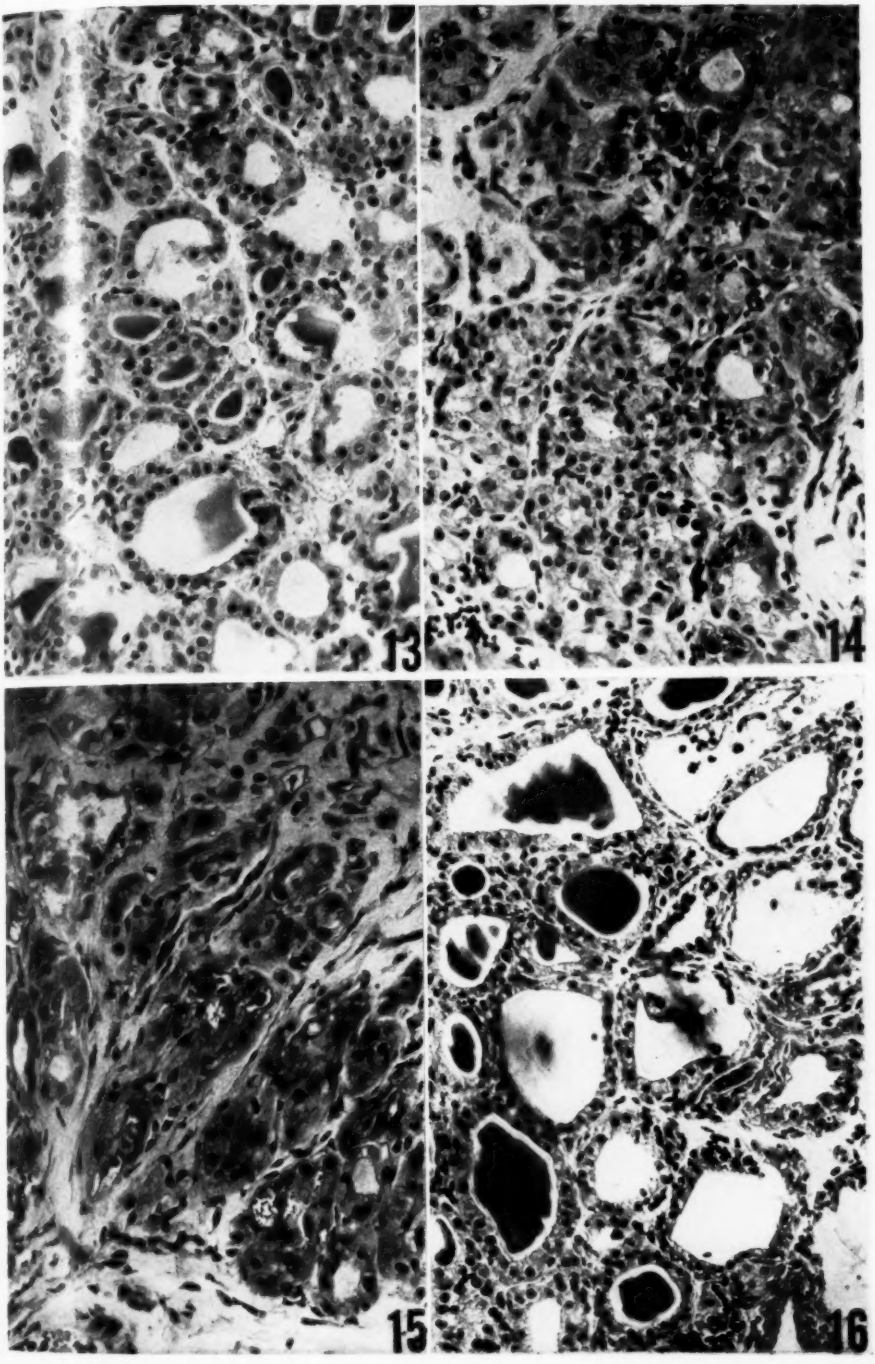
PLATE 40

FIGURE 13.—Typical thyroid gland in control mouse on diet #77. *Note* slightly elevated epithelium surrounding follicles. Gomori-trichrome. × 270

FIGURE 14.—Thyroid gland of mouse 48 days after it received a subcutaneous injection of 30 μc of I^{131}. A few small follicles are present. Much disorganization of the tissue is present.

FIGURE 15.—Thyroid gland of mouse 48 days after a subcutaneous injection of 30 μc I^{131}, and 34 days after inoculation with tumor 3C. Very few follicles remain and extensive amounts of fibrous tissue are present.

FIGURE 16.—Thyroid gland of mouse treated for 33 days with exogenous TSH. The histologic structures appear essentially normal.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16

The Action of *N*-2-Fluorenylacetamide in the Syrian Golden Hamster [1,2]

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Summary

N-2-Fluorenylacetamide was fed at a 0.04 percent level in the diet to female and male Syrian golden hamsters for 40 or 26 weeks. A high incidence of benign cholangiomatous lesions and some cholangiocarcinomas were seen. Outside of the liver, only 1 colonic adenocarcinoma, 1 leiomyosarcoma, and 1 generalized lymphoma were observed that were not believed to be related to the experimental diet. No parenchymal liver-cell tumors were observed. No definite differences between the male and female response were noted. The pathological findings are discussed in the light of their histopathogenesis.—**J. Nat. Cancer Inst. 22: 463–487, 1959.**

This study records the effect of *N*-2-fluorenylacetamide in the Syrian golden hamster. *N*-2-Fluorenylacetamide is one of the most versatile carcinogens known and has been tested in many species (*1*, *2*). So far, only one brief study in the hamster by Paschkis *et al.* (*3*), who administered the carcinogen simultaneously with diethylstilbestrol to female hamsters and induced cholangiomas, has been reported.

Materials and Methods

Female and male Syrian golden hamsters (Abram Small Stock Breeders, Chicago), 10 to 12 weeks old at the beginning of the experiment, were used. They were housed in plastic cages in groups of 5, according to sex, and given food and tap water *ad libitum*. The experimental diet consisted of powdered Rockland mouse diet mixed with 0.04 percent *N*-2-fluorenylacetamide (AAF). When this diet was withdrawn, the animals were fed Rockland mouse diet in pellets. Each animal consumed an average of 50 gm. of food per week and thus received approximately 20 mg. of AAF weekly.

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Two experiments were performed. In the first experiment, 15 females and 15 males were used and the experimental diet was fed continuously for 40 weeks; the survivors were then shifted to the normal pellet diet. In the second experiment, 10 males and 10 females were fed the experimental diet for 26 weeks and then the survivors were fed the normal pellet diet. The animals were allowed to die spontaneously or were killed with ether when in poor condition. Pathological study was undertaken on all animals with the exception of a few animals that were lost because of cannibalism. Histologic examinations were usually made of tissues fixed in 10 percent buffered formalin, mounted in paraffin, and stained routinely with hematoxylin and eosin or with special staining techniques when needed. Occasionally, Carnoy, Bouin, and Zenker fixatives were used.

Several groups of hamsters served as controls; they were obtained at 8 to 10 weeks of age from the same source as the experimental animals. Most animals were kept until spontaneous death occurred or were killed when moribund; some were killed at a given time. A total of 82 females and 69 males was available for pathological study.

Results

First Experiment

In the female group, 1 animal died at the 5th week, 4 at the 14th, 6 at the 23rd, 1 at the 29th, 1 at the 33rd week, and the last 2 died at the 39th week from the beginning of the experimental diet.

In the male group, 7 animals died or were killed at the 23rd week and 1 at the 29th week. At the 40th week the experimental diet was stopped, and the 7 male survivors were fed the basal diet in pellets. Three animals died or were killed when moribund at the 50th, 57th, and 62nd week, respectively, 2 at the 69th, and 1 at the 75th week. The last male died at the 84th week from the beginning of the experiment.

Autopsy of the females dying at the 5th and 14th weeks showed bronchopneumonia and no other gross pathological findings. Microscopic study showed acute congestion and a diffuse, mild, fatty metamorphosis of the liver; in the portal spaces large histocytes laden with a brownish granular pigment were noted. Most of this pigment gave a positive iron reaction. Some of it was PAS-positive and some of the coarser granules stained deeply red with Oil Red O in paraffin section. In the portal spaces there was a moderate accumulation of bile-duct cells, arranged in small clusters or in double rows without evidence of a lumen; they were associated with scanty fibroblastic proliferation and a few mononuclear inflammatory cells.

Gross examination of the liver of the 6 females and 7 males dying during the 23rd week showed no abnormalities, except in 1 female and 1 male in which the liver showed multiple, small cystic structures measuring up to 3 mm. in diameter and filled with a watery, light-green fluid. This cystic change was more marked in the female hamster, the liver appearing

moderately enlarged and distorted. Histologic examination did not show a consistent picture. Fatty changes were seldom seen but when present they were predominantly at the periphery of the lobules. In the portal spaces bile-duct-cell proliferation and pigmented macrophages were constantly present, occasionally associated with mild fibrosis. Among the irregularly arranged epithelial cells and those forming pseudotubules, there were newly formed luminized ducts with the histologic characteristics of bile ducts (fig. 1). Some of them appeared dilated to form small cysts lined by a cuboidal epithelium (fig. 2). These cysts were mostly empty or contained a finely granular material, but occasionally they were filled with blood or with a polymorphonuclear leukocytic exudate. In the male, in which the cystic structures were grossly seen, slides showed the cysts to be lined by a flattened epithelium which assumed an endothelium-like appearance. In the female dying at the same time, the lesion was more advanced with more numerous and larger cysts and distortion of the lobular pattern. The cysts were either in direct contact with the liver cords, with little stroma intervening, or they were surrounded by abundant fibrous tissue. The biliary proliferation often appeared to compress and invade the periphery of the liver-cell cords. Degenerating liver cells frequently were seen between the newly formed ducts. The liver cells of the intact lobules were usually well preserved. A few of them, scattered irregularly throughout the lobule, had rather large nuclei, each containing 1 and occasionally 2 bodies with a sharp basophilic membrane incorporating a delicate granular eosinophilic material (figs. 3 and 4). These intranuclear bodies were of varying size and, on occasion, large enough to occupy the nucleus almost completely. In the nuclei there were one to several prominent nucleoli.

The 2 animals dying at the 29th week were lost for pathological study because of cannibalism. The last 3 females dying between the 33rd and 39th week showed marked enlargement and distortion of the liver, which had a multicystic appearance. On histologic examination the liver architecture was greatly obliterated by cystic structures of varying sizes. Most of them were lined by a flattened epithelium and were packed together forming a spongy structure with little intervening stroma and small strips of disorganized parenchymal tissue scattered throughout. In focal areas, small groups of cysts had a more definite glandular pattern; they were composed of a cuboidal or low-columned epithelium showing a few mitotic figures, but no obvious atypicalities. Particularly in the left lobe there were large areas of parenchyma, arranged in a normal lobular pattern, showing no significant changes of the liver cells.

Seven males survived at the end of the AAF diet. One of them was killed as a control 10 weeks later at the 50th week from the beginning of the experiment; histologic sections of the liver showed marked proliferation of bile-duct epithelium and numerous newly formed bile ducts, but these changes were much less prominent than those seen in the 3 females that died between the 33rd and 39th week. The lobular architecture was generally well preserved, although there were focal necrosis and fatty

changes in the perilobular areas. The intranuclear bodies previously described frequently were seen. Another male was lost because of cannibalism at the 57th week.

The 5 remaining males died between the 62nd and the 83rd week. Their livers were very enlarged and distorted by numerous cysts. The solid areas frequently showed hemorrhages. Histologic study revealed large aggregates of cysts, mostly lined with a flattened epithelium and packed together to form a multilocular pattern (fig. 5). In many areas these cysts were in direct continuity with glandular structures lined by a cuboidal epithelium and showing an adenomatous pattern (fig. 6). Very often the same glandular structure was lined partly by a flattened endothelium-like epithelium and partly by a cuboidal or columnar epithelium. Occasionally the glands were branched or showed papillary projections or both (fig. 7). Sometimes they were embedded in abundant connective tissue in which a few hepatic cells were included. Rarely, there was a marked inflammatory infiltration, often with suppuration into the lumen (fig. 8). The cuboidal and columnar epithelium had rounded or oval nuclei with a fine network of deeply basophilic chromatin and small nucleoli. The nuclei were regularly arranged in a single layer at the base of the cells, while the cytoplasm protruded into the lumen and produced minute droplets of protein-like material, which were negative with the mucicarmine stain. Mitoses were rarely seen. In 2 of these 5 males, dying at the 62nd and 69th week, respectively, evidence of frank adenocarcinoma was demonstrated in association with the lesions described. These 2 carcinomas were composed of small and large glandular structures lined by an irregular epithelium (figs. 9 and 10). The cytoplasm was pale to deeply acidophilic. Nuclear chromatin was irregularly arranged and mitoses were frequent. In one of them, there were numerous goblet cells and the glandular lumens contained large amounts of mucinous material, which was well demonstrated by the mucicarmine stain (figs. 11 and 12). No metastases were observed. In all 5 animals, the liver parenchyma was greatly distorted and was destroyed by compression from the cystic conglomerate and by actual invasion and disruption of the lobular architecture by the actively growing adenomatoid proliferation. Often areas of hemorrhagic infiltration and necrosis were seen. The hepatic cells in the preserved areas presented variation in size and shape of the nuclei, which contained large nucleoli or the previously described intranuclear bodies, but no neoplastic changes were seen. The gallbladder never showed any remarkable abnormality.

In this first experiment, only one tumor was seen outside of the liver. This was a mucus-producing, infiltrating adenocarcinoma of the cecum in 1 of the 2 male hamsters that died at the 69th week and which did not have an intrahepatic adenocarcinoma. There were no metastases. Gross and microscopic studies of the other organs failed to reveal any major abnormality that could be related to the AAF diet. Intestinal infections frequently were seen. The spleen showed moderate fibrosis in the late stages of the experiment. No hyperplastic changes were observed in other organs or tissues.

Second Experiment

In this experiment, all 10 animals in the female group were still alive at the end of 26 weeks of the AAF diet. They were in good condition. The body weight was slightly decreased, the mean value being 106 gm. instead of 113 gm., as at the beginning of the experiment. At the 30th week 1 animal died and was lost because of cannibalism. At the 44th week 1 had a very enlarged abdomen and was killed for a control. At the 50th week all the 8 females surviving had an enlarged abdomen; by palpation the liver was found to be greatly enlarged, the average weight was 121 gm., and the general condition was poor. No jaundice was noted. Another animal died at the 62nd week, and the last 3 animals were killed when moribund at the 63rd, 67th, and 68th week from the beginning of the experiment. The weight of these 4 animals ranged from 205 to 297 gm. at the time of death.

At autopsy, all the 9 females available for pathological study had extremely enlarged livers that weighed from 65 to 100 gm. and had been transformed into a multicystic mass (fig. 13). Some of the cysts measured up to 1.5 cm. in diameter, were under tension, and contained a watery, yellowish fluid. On cut surface, the dilated cysts were interspersed with smaller areas of a spongy tissue and with a few strips of a solid tissue which often appeared hemorrhagic. These solid areas were present particularly in the posterior portion of the left lobe. The gallbladder was small and contained light bile. The spleen was generally rather small. In the peritoneal cavity there were a few milliliters of reddish fluid. No other gross abnormality was seen. The microscopic study showed essentially the same picture as that seen in the advanced lesions in the course of the first experiment. The larger cysts were lined by a flattened epithelium. The spongy areas were composed of similar smaller cysts and glandular structures, surrounded by fibrous tissue or packed together in an adenomatous pattern, which occasionally showed papillary projections. Although in this entire series of animals marked histological and cytological irregularities of the glandular formations were often observed, a diagnosis of adenocarcinoma was made only in 2 animals. One of these was seen in the female killed at the 63rd week; it was composed of large and small glandular structures. The second was present in the animal killed at the 67th week. It was composed of small, irregular glands actively invading the liver cords (fig. 14). There were no metastases. The areas that grossly had a solid appearance were composed of remnants of liver parenchyma with greatly distorted architecture. Very few preserved lobules were recognizable. As in the first experiment, most of the surviving parenchymal cells showed marked degenerative changes except in a few areas in which a regenerative process with numerous mitoses was evident. Occasionally nuclear irregularities were present, but the histologic picture was not suggestive of a neoplastic proliferation of parenchymal cells. As in the previous group, several eosinophilic intranuclear bodies were found. Histologic studies of other organs and tissues failed to reveal any remarkable pathological change, except for moderate splenic fibrosis. In three

instances, transplantation of randomized fragments of the cystic liver was attempted unsuccessfully.

In the male group of the second experiment the average weight was 88 gm. at the beginning of the experimental diet. One animal died after 2 weeks; histologic examination showed early necrosis of the liver parenchyma in the perilobular areas. A 2nd animal died at the 5th week. Sections of the liver showed a well-preserved lobular architecture; in the portal spaces there were mild proliferations of bile-duct epithelial cells and histocytes carrying brown pigment. No alteration of the parenchymal cells was observed. At the 26th week, at the end of the AAF diet, the 8 survivors were in good condition and weighed an average of 101 gm. The weight reached the mean value of 110 gm. at the 50th week. At the 54th week 1 animal was lost because of cannibalism. At the 60th week the average weight of the 7 survivors was 112 gm. Their general condition was good; no abdominal enlargement was noted. Another animal died at the 67th week, 2 at the 68th, and a 4th died at the 72nd week. This last animal weighed 140 gm. at the time of death. At the 80th week the last 3 animals were in good condition and were killed. Their weight ranged from 115 to 130 gm.

At autopsy, of the 7 males that died or were killed between the 67th and the 80th week, the liver showed essentially the same picture. The weight ranged from 20 to 30 gm. The left lobe was the most enlarged; it was composed of cysts measuring up to 1 cm. in diameter but predominantly it consisted of solid hepatic tissue. The right lobe was moderately enlarged and completely replaced by small cystic structures. The posterior lobes were partially cystic. The major deviation from this general gross appearance was present in the animal that died at the 67th week; the liver showed a complete replacement of all the lobes by a mixture of spongy and lardaceous tissue. Histologic studies of this liver revealed a total obliteration of the normal architecture by a glandular proliferation composed of large, irregular glands with columnar epithelium surrounded by a loose abundant stroma. No cystic dilatation of these glands was present. Small areas of liver cells with evidence of degeneration were scattered through the lobes. In occasional focal areas the spreading of glandular proliferation assumed the appearance of well-circumscribed cholangioadenomas (fig. 15). In addition, multicentric small foci diagnostic of adenocarcinoma were seen. Sections from the remaining 6 animals showed essentially the same features described in the female group, although they differed in some details. The cystic structures were mostly small, and the glandular formations had more abundant stroma. In all 6 animals, multifocal areas showed a high degree of irregularity within the adenomatoid proliferation (fig. 16), but in none could a diagnosis of cholangiocarcinoma be made with certainty. No adenomatous nodules or tumors of hepatic-cell origin were seen, although in the animals killed at the 80th week regenerative activity with nuclear variations was present. Occasionally intranuclear bodies were seen. Outside of the liver there were 2 other tumors. One animal that died at the 72nd week

had a generalized lymphoma that could not be classified because of *post-mortem* changes. In addition, 1 of the animals that was killed at the 80th week had a large leiomyosarcoma apparently arising in the retroperitoneal area at the site of the mesenteric lymph nodes. No other significant abnormality was present elsewhere.

Controls

Ten females and 3 males died between the 23rd and 40th week of age, 18 and 9 between the 41st and 60th, 28 and 16 between the 61st and 80th, 3 and 23 between the 81st and 100th, and 16 males between the 101st and 120th week of age. In addition, 10 females and 15 males were killed between the 41st and 60th week. Pathological examination showed 1 mucus-producing cholangiocarcinoma in 1 female dying at 26 weeks of age. No other lesions were seen in the animals that died or were killed before the 60th week, but in 6 females dying between the 42nd and the 60th week that had 1 to 4 cysts, measuring from 1 to 5 mm. in diameter, small groups of microcysts and a moderate proliferation of bile-duct cells were seen; in addition, in 2 of the 6 animals there were scattered areas of cholangiofibrosis. In the remaining 31 females and 55 males surviving after the 61st week no liver tumors were seen. Small conglomerates of cysts, usually located at the margins of the liver and measuring 5 to 10 mm. in diameter, were observed in approximately 80 percent of these animals, with no sex difference. The cause of death of most of these animals was found to be an intestinal infection of unknown origin.

Discussion

The results of the two experimental groups are essentially similar. Histopathologic study revealed the following series of lesions of the bile ducts: proliferation of epithelial cells with formation of pseudotubules, hyperplasia and cystic dilatation of bile ducts, cholangioadenomas, and cholangiofibrosis. In five instances, in 2 females and 3 males, a cholangioadenocarcinoma was present. No significant difference between the two sexes was noted except that the females developed the more advanced cystic lesions earlier. The liver parenchyma was extensively destroyed by the biliary lesions and generally behaved in a passive way. No liver-cell adenomatous nodules or carcinomas were seen, although in the animals that died or were killed in the late stages of the experiment regenerative changes with abnormalities of the liver cells were present. In the liver cells, large intranuclear eosinophilic bodies frequently were seen that were similar to those described by Schoental and Magee (*4*) in the liver of rats fed lasiocarpine. In control animals they were occasionally observed. The significance of these intranuclear bodies is not clear. No cytoplasmic inclusions, as described by Price *et al.* (*5*), in the liver of rats fed azo dyes nor hyaline bodies like those seen by Edwards and White (*6*) were found. Both hemosiderotic and ceroid pigments were always present. The architectural distortion characteristic of true cirrhosis was not seen

in this study, though there were small and large areas of fibrosis surrounding glandular structures and presenting the characteristic picture of cholangiofibrosis (*7*).

The cholangiomatous lesions found in our hamsters seem to be similar to those described by many investigators as occurring in the rat during liver carcinogenesis with a variety of chemicals. These lesions, and their relationship to carcinogenesis in the rat liver generally, are critically reviewed by Stewart and Snell (*8*). In the hamster, under our experimental conditions, we did not observe any liver-cell tumors. This fact seems to be worthy of note, particularly because Stewart and Snell emphasized their belief that liver tumors may originate in a unitary way from the hepatic parenchymal cell. In the present study we observed a sequential series of lesions that started from a simple bile-duct hyperplasia and eventuated in cholangioadenomas and cholangiocarcinomas. Thus, in the hamster, it seems to be possible to have a line of tumor formation independent of the liver parenchymal cells.

In other studies and in control animals we observed a rather high incidence of the bile-duct hyperplasia and cystic dilatation and a few cholangiocarcinomas not related to a known extrinsic factor. This is confirmed by the findings of Fortner (*9*, *10*). In this study, therefore, AAF has essentially enhanced the incidence of a commonly occurring neoplasm. If this action is to be classified as carcinogenic, it must be equated with other well-known situations such as the enhancement of lung adenoma induction in a strain of mice with a naturally high incidence of such tumors (*11*). Most investigators have not, however, distinguished between such enhancements and the induction of a type of tumor not seen commonly in the untreated animal. An examination of this general problem is clearly complex and beyond the scope of the present investigation.

In the rat, in addition to the liver tumors, AAF is known to induce a broad spectrum of neoplasms involving different organs and tissues (*12*, *13*). In our experiments we observed only 1 colonic adenocarcinoma, 1 leiomyosarcoma, and 1 generalized lymphoma that appeared to be unrelated to the experimental diet in view of the reported spontaneous incidence of such tumors in the hamster (*9*, *10*).

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PLATE 41

FIGURE 1.—Bile-duct hyperplasia. Hematoxylin and eosin. × 120

FIGURE 2.—Hyperplastic and dilated bile ducts. Hematoxylin and eosin. × 340

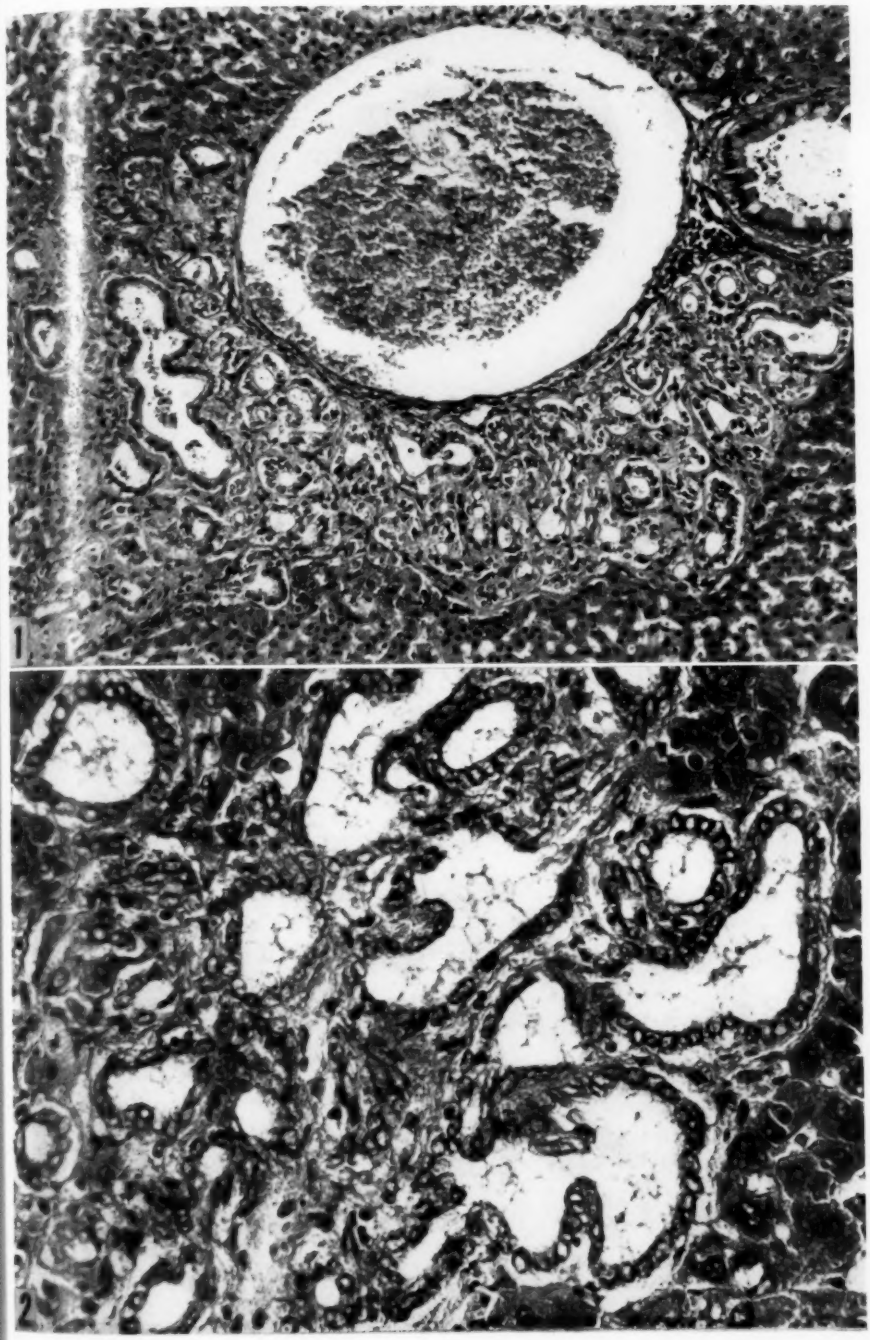
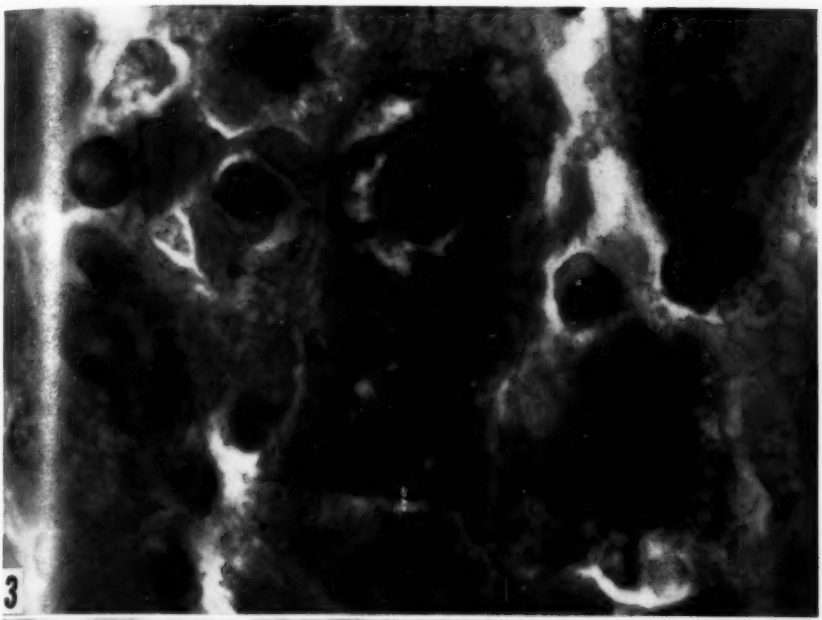


PLATE 42

FIGURE 3.—Enlarged liver cells containing eosinophilic intranuclear bodies. Hematoxylin and eosin. × 2,000

FIGURE 4.—Enlarged irregular nucleus of liver cell containing eosinophilic intranuclear body and several prominent nucleoli. Hematoxylin and eosin. × 3,000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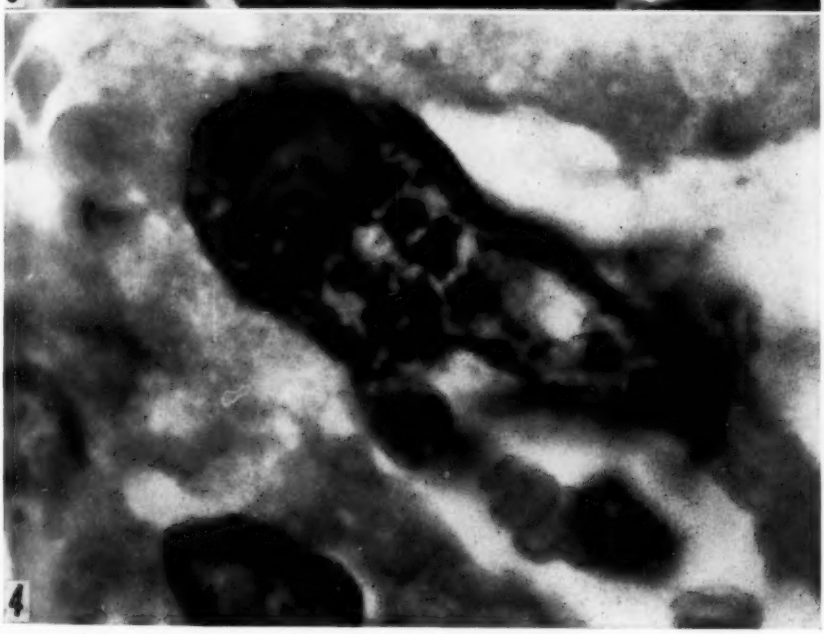
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PLATE 43

FIGURE 5.—Replacement of the liver structure by cysts mostly lined by a flattened epithelium. Hematoxylin and eosin. × 30

FIGURE 6.—Cystic and glandular structures. Hematoxylin and eosin. × 150

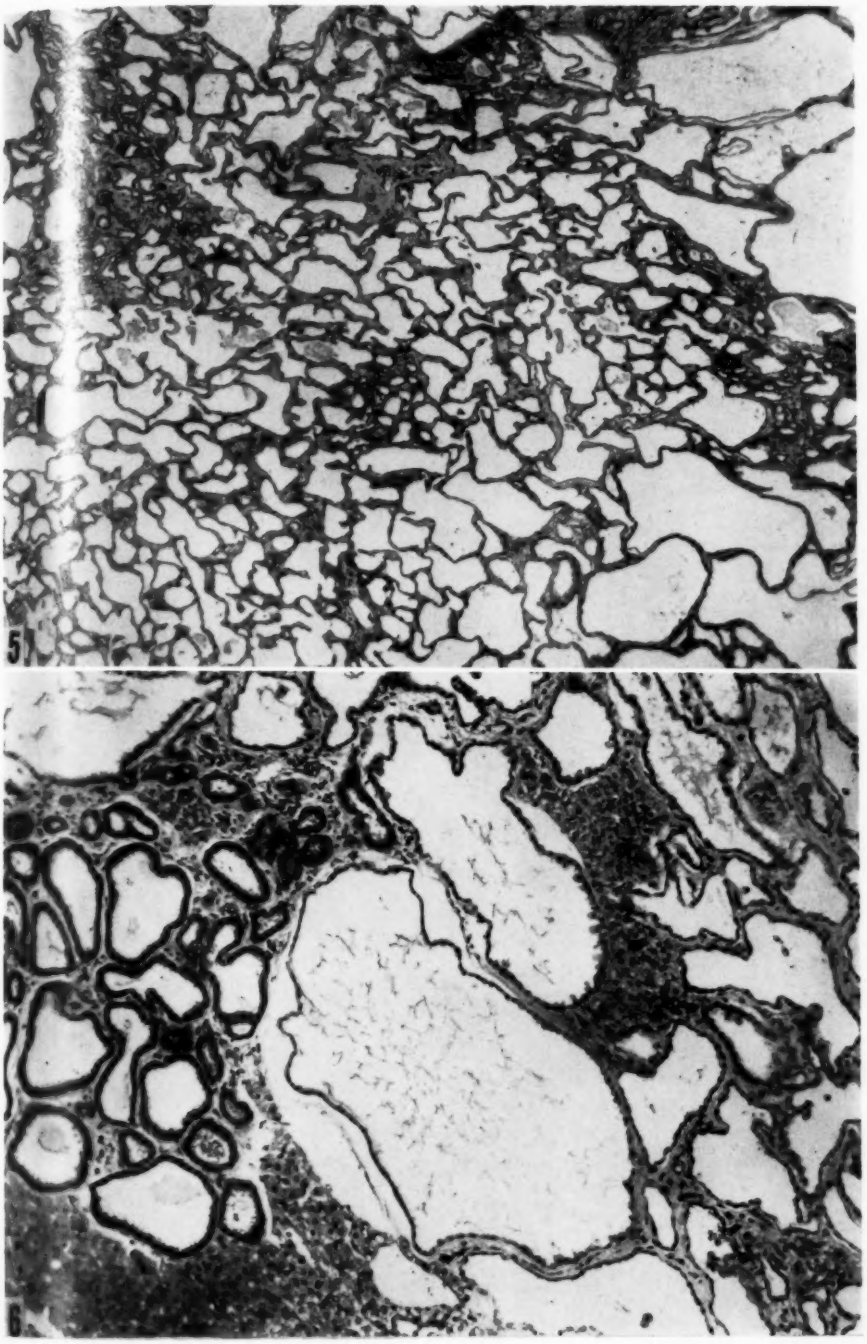

PLATE 44

FIGURE 7.—Branched glands with papillary projections. Hematoxylin and eosin. × 180

FIGURE 8.—Suppurative exudate in hyperplastic, dilated bile ducts. Hematoxylin and eosin. × 100

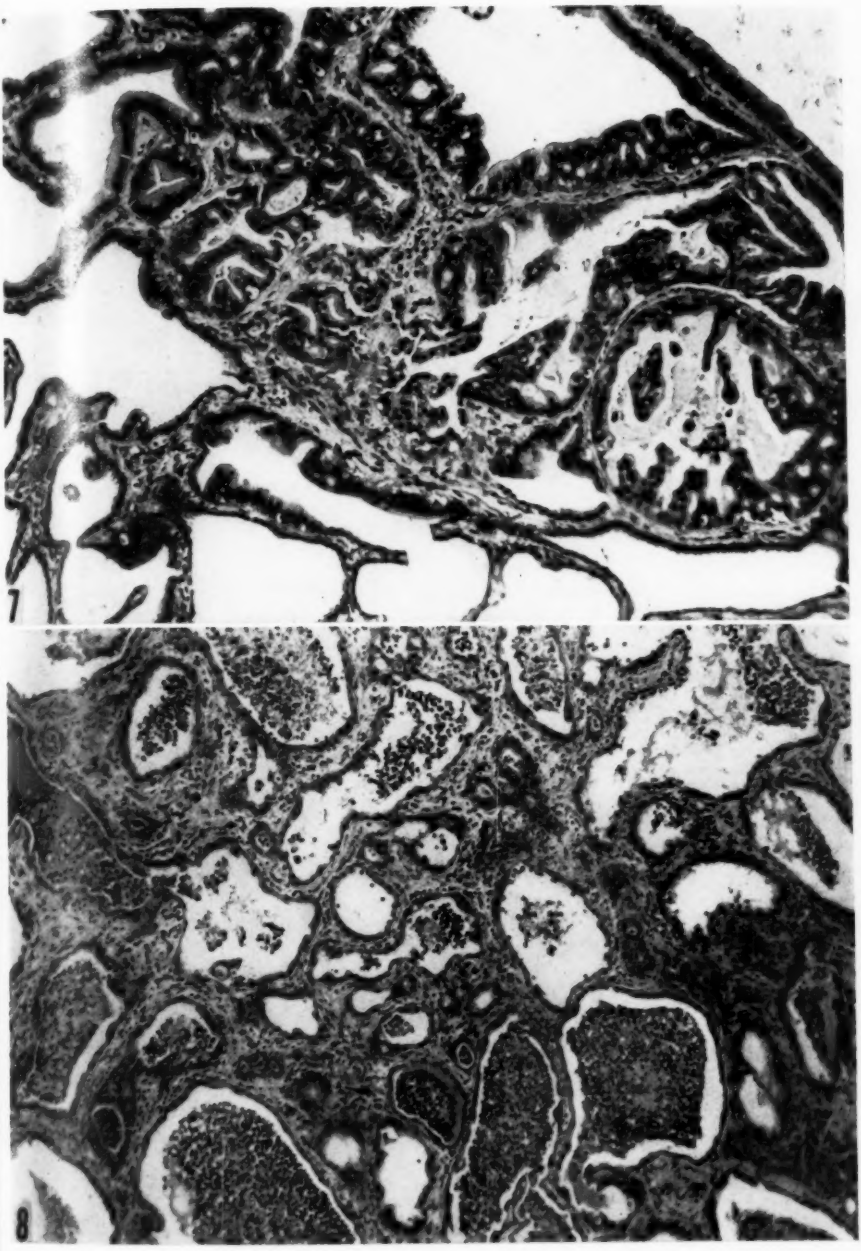

PLATE 45

FIGURE 9.—Extensive neoplastic bile-duct proliferation with abundant stroma. Remnants of liver parenchyma are present. Hematoxylin and eosin. × 40

FIGURE 10.—Higher magnification of the same area as figure 9, showing carcinomatous glands. Hematoxylin and eosin. × 250

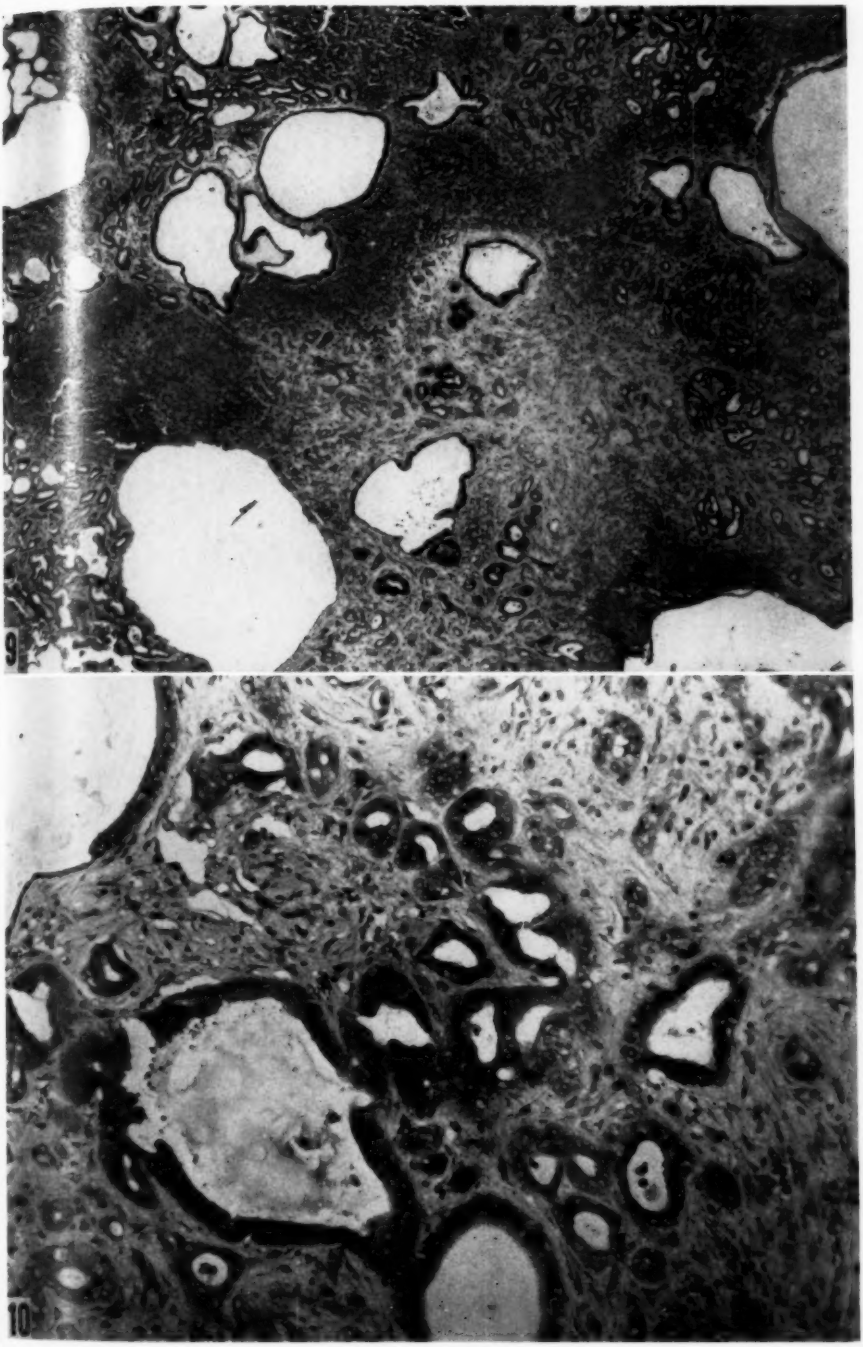

PLATE 46

FIGURE 11.—Large glandular structures containing abundant mucus in continuity with small carcinomatous glands. Hematoxylin and eosin. × 80

FIGURE 12.—Higher magnification of figure 11, cholangiocarcinoma. Hematoxylin and eosin. × 250

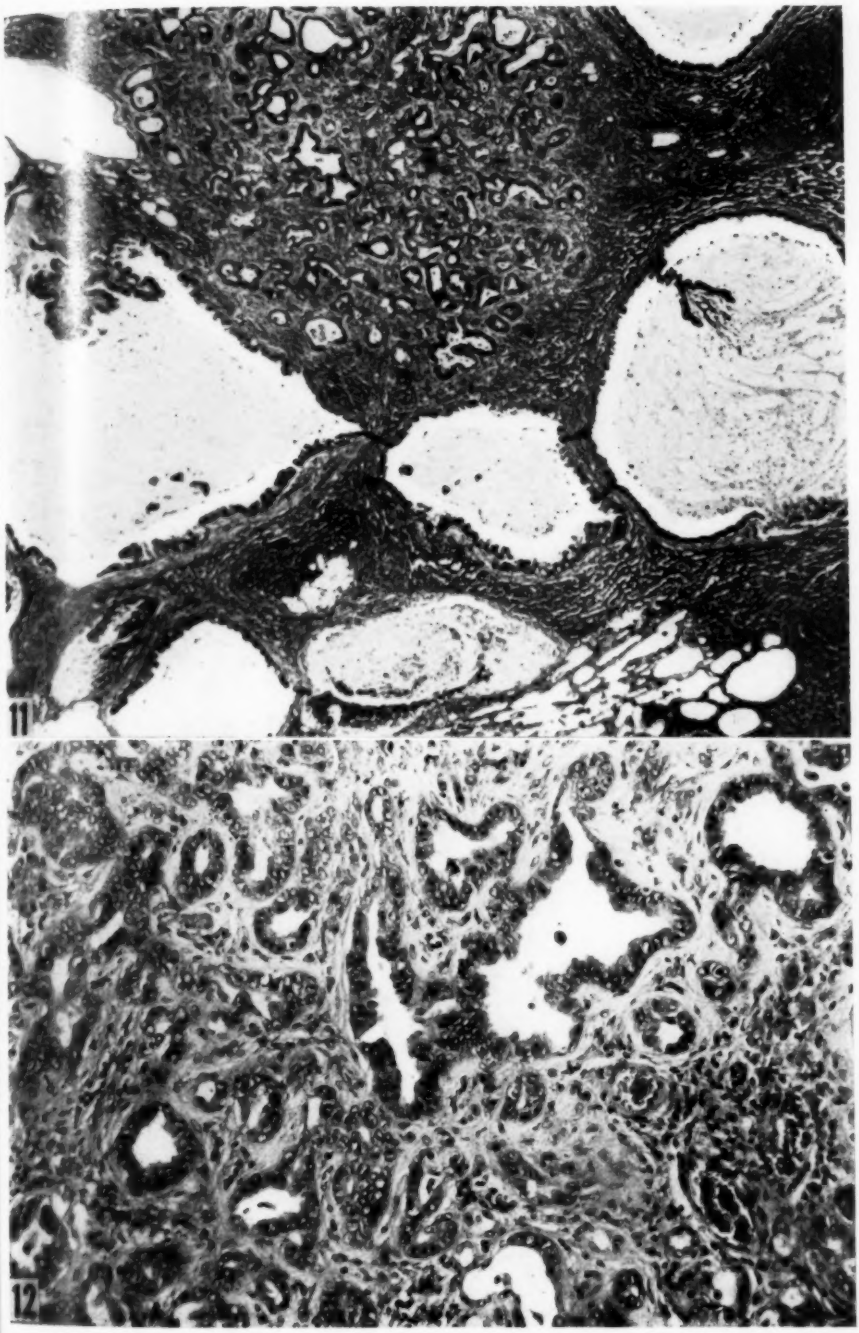
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PLATE 47

FIGURE 13.—Liver, markedly enlarged and transformed in a multicystic mass.

FIGURE 14.—Small glands of carcinoma infiltrating the liver cords. Hematoxylin and eosin. × 340

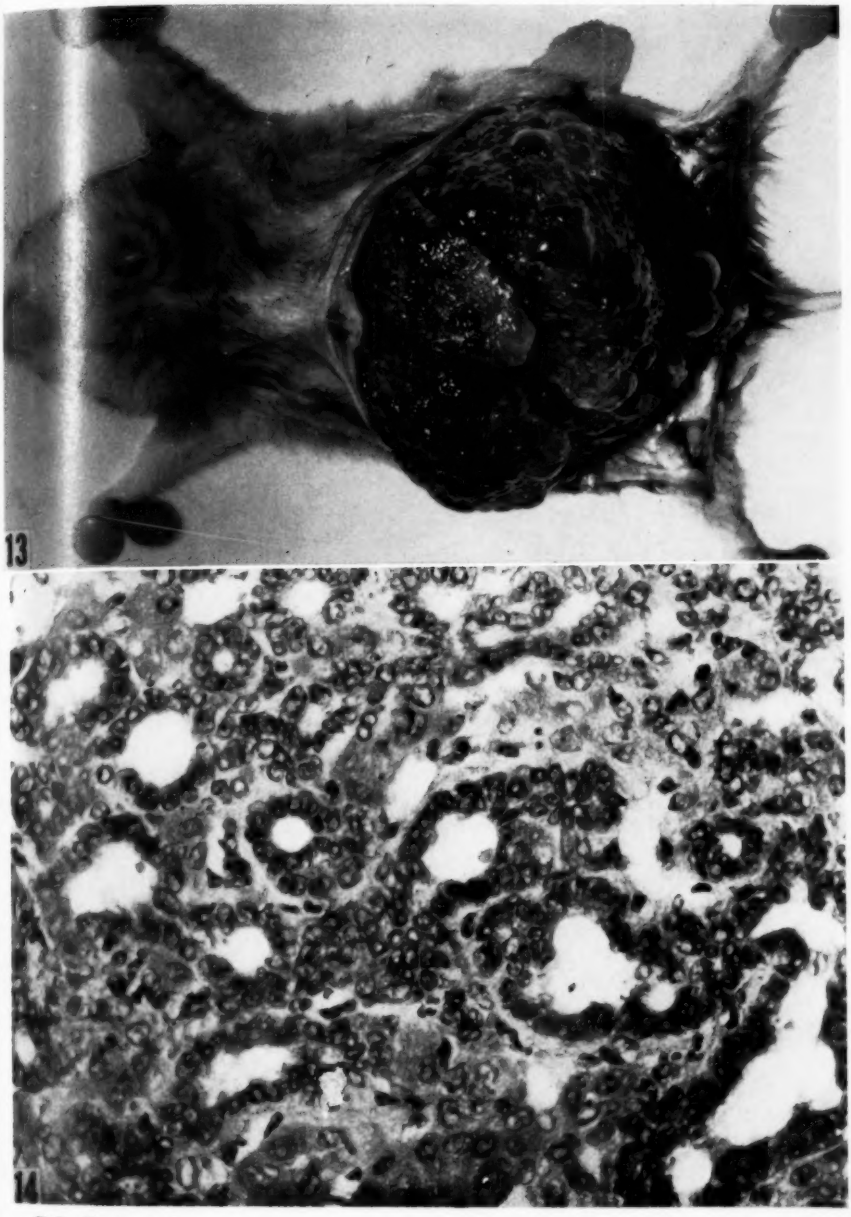
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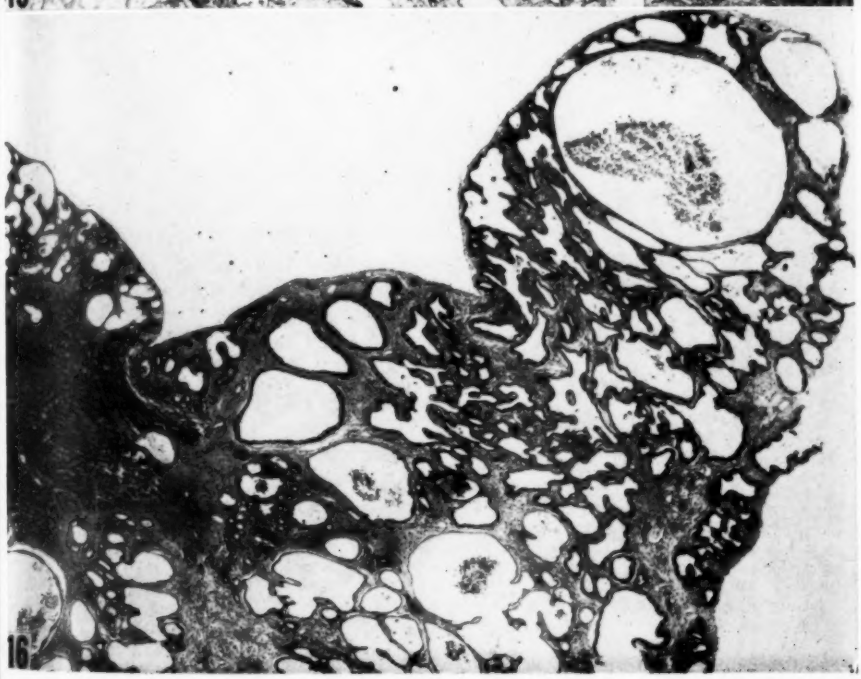
PLATE 48

FIGURE 15.—Well-circumscribed cholangioadenoma with glands containing large amounts of amorphous proteinic material. Hematoxylin and eosin. × 45

FIGURE 16.—Margin of the liver replaced by dilated glands mostly lined by tall, deeply stained epithelium. Hematoxylin and eosin. × 36



493212—59——5

Effect of 5-Fluorouracil on Mammary Tumors in Mice [1,2]

ELIZABETH M. CRANSTON,[3,4] *Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota*

Summary

5-Fluorouracil prevented growth of second- to fourth-generation transplants of a mammary adenocarcinoma in mice. On cessation of therapy or transplantation of treated tumors into untreated animals, growth of tumors occurred. Results obtained in mice with spontaneous tumors suggest that very occasionally 5-fluorouracil may cause regression of such tumors.—**J Nat. Cancer Inst. 22: 489–495, 1959.**

The fluorinated pyrimidines have been shown to be active against a variety of tumors and against several microorganisms (*1–4*). The present paper reports results obtained with one of these agents, 5-fluorouracil, in mice with transplants of recent isolates of a mammary adenocarcinoma and in a few mice with spontaneous tumors.

Methods

In most experiments a spontaneous mammary adenocarcinoma, arising in a Z female breeding mouse, was transplanted aseptically by trocar into young ZBC [5] female mice and retransplanted into other female ZBC mice for later generations. Transplants were made into the axillary region. The second through fourth generations of the same tumor were used in these experiments. Mice were divided between control and treated groups on the basis of approximately equal size of tumors.[6] Therapy with 5-fluorouracil was usually begun when tumors measured approximately 1.5 × 2.0 mm. (10 to 14 days after transplantation) and

[1] Received for publication August 22, 1958.

[2] This work was supported by grant CY-1962 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] With the technical assistance of Kenneth M. Hanson.

[4] The author wishes to express her gratitude to Dr. M. J. Schiffrin, of Hoffman-LaRoche, Inc., Nutley, N.J., for supplies of 5-fluorouracil; to the late Dr. J. S. McCartney, Department of Pathology, University of Minnesota Medical School, for the pathological studies; and to Dr. R. N. Bieter, Department of Pharmacology, and Dr. J. J. Bittner, Division of Cancer Biology, Department of Pathology, University of Minnesota Medical School, for the time and guidance they have so willingly given.

[5] Z mice are C3H mice. ZBC mice are obtained as follows: A × Z = AZ F_1, AZ F_1 × Z = ZBC.

[6] Tumors were measured with calipers in two directions at right angles to one another. Measurements of small tumors (1.5 × 2 mm.) are approximate values.

493212—59——6

TABLE 1.—*Effect of 5-fluorouracil on weight of transplants of a mammary adenocarcinoma in mice*

Dose, intraperitoneally (*mg./kg.*)	Number days therapy	Number of mice	Change body weight* (*gm.*) Treated / Control	Tumor weight† (*gm.*) Treated	Tumor weight† (*gm.*) Control	Percent change in tumor weight	Number deaths / Number treated
40	8–9						10/10
30	9–14						10/10
25	14	13	−3. 3/+2. 1	. 0111 ± . 0038	. 1859 ± . 0391	−94. 0‡	2/15
20	14	28	−0. 6/+2. 1	. 0181 ± . 0050	. 1324 ± . 0311	−86. 3‡	2/30
20§	14	12	−2. 2/+0. 7	. 2348 ± . 0393	. 5373 ± . 0946	−56. 3‡	0/12
20‖	14	6	−0. 3/0	. 6663 ± . 1026	1. 6463 ± . 3577	−59. 5¶	0/6
10	14	20	+1. 1/+2. 0	. 0381 ± . 0077	. 0766 ± . 0202	−50. 3	0/20
5	14	10	+1. 9/+2. 0	. 0865 ± . 0120	. 0766 ± . 0202	+12. 9	0/10
25–20**	28	18	+2. 2/+1. 0	. 0020 ± . 0004	. 0625 ± . 0102	−96. 8‡	12/30

*Change in body weight means the difference between weight of mouse with tumor at start of experiment and weight of mouse minus tumor at end of experiment.
†Tumor weight ± the standard deviation of the means.
‡*P* value is less than 0.01.
§Tumors measured 6.3 × 7.7 mm. at start.
‖Tumors measured 12.0 × 16.8 mm. at start.
¶*P* value is approximately 0.03.
**Twenty-five mg. per kg. for 14 days, then 20 mg. per kg. for 14 days.

continued for 14 days. In one experiment, therapy was started when tumors were larger; in another, it was begun 3 days after transplantation, at which time the tumors were not palpable, and continued for 28 days. A few mice with spontaneous tumors were also treated.

5-Fluorouracil was dissolved in saline and administered daily by intraperitoneal injection, 6 times a week, in a volume of 0.1 ml. per 20 gm. mouse. Mice were housed 5 to a cage and fed Purina fox chow and water *ad libitum*. Control mice received no injections. At autopsy, on the day after the last injection, tumors were dissected out and weighed on a Roller-Smith precision balance.

Results

Table 1 shows that administration of 20 and 25 mg. per kg. of 5-fluorouracil daily for 14 days caused marked inhibition in the weight of tumors. Only a slight loss in body weight occurred with 20 mg. per kg. A greater loss was seen with 25 mg. per kg. but this, *per se*, was insufficient to account for the marked inhibition in tumor weight.[7] Higher doses were lethal to mice and lower doses were without significant effect on tumors. When therapy was started 3 days after transplantation and continued for 28 days, a marked inhibition in tumor weight was also obtained. At the time of autopsy of these latter mice, tumors could not be palpated in 15 of 18 treated mice, but a tumor was found in all animals. Thus the drug prevented growth but did not cause dissolution of the tumor. Twelve of the original 30 mice on therapy in this experiment died due to drug toxicity. Table 2 shows that inhibition occurred regardless of size of tumors at the start of therapy and was due to an almost complete suppression of growth. Tumors measured approximately the same at the end of treatment as at the beginning.

Pathological studies of tumors suggested a possible slight increase in fibrosis after 14-day therapy with 5-fluorouracil. No other change was noted.

TABLE 2.—*Effect of 5-fluorouracil on the growth of transplants of a mammary adenocarcinoma in mice**

Treatment (*mg./kg.*)	Average diameter of treated tumors (*mm.*)		Average diameter of control tumors (*mm.*)	
	Start	2 Weeks	Start	2 Weeks
25	1.7 × 2.0	1.5 × 1.9	1.8 × 2.1	5.5 × 6.5
20	1.5 × 1.7	1.5 × 1.8	1.4 × 1.8	4.5 × 5.6
20	6.3 × 7.7	7.2 × 7.8	6.4 × 7.6	9.5 × 10.4
20	12.0 × 16.8	13.0 × 17.1	10.7 × 16.3	16.5 × 23.8

*Lines 1, 2, 3, and 4 in this table are data from mice constituting the results found in lines 3, 4, 5, and 6, respectively, in table 1.

[7] In an experiment using untreated mice on control diets that were restricted in amount to varied food intakes, the following data were obtained: 40 percent inhibition in tumor weight with a 3 gm. loss of body weight, 68 percent tumor inhibition with a 5 gm. loss of body weight, and 96 percent tumor inhibition with a 7 gm. loss of body weight. This indicates a 14 percent inhibition in tumor weight per gram of body weight lost on restricted control diets.

Table 3 gives data obtained in mice treated with 5-fluorouracil for 14 days. At the end of this time tumors were removed and transplanted into untreated recipient mice. It is evident that, though therapy stopped the growth of tumors in donor mice, treated tumors grew when transplanted into untreated mice.

In another experiment (table 4) mice were treated with 5-fluorouracil for 14 days and then observed for another 12 weeks without further treatment. Tumors became smaller during treatment and could not be palpated in 2 mice at the end of therapy. However, growth started in all mice after cessation of therapy. During the weeks following treatment the treated tumors at any given time were smaller than control tumors due to the difference in size at the end of therapy, but the rate of growth in the 2 groups was approximately parallel.

Twenty-eight mice with spontaneous tumors were treated with 5-fluorouracil daily until death, except where otherwise noted. Twelve mice were given 25 mg. per kg. for the first 2 to 4 weeks and then 20 mg. per kg., the higher dose being too toxic. The rest received 20 mg. per kg. throughout therapy. Control mice were not used because the availability of mice with spontaneous tumors was extremely limited. It seemed desirable to try the drug against such tumors in spite of this lack. Twenty-seven mice were Z breeding females. In 23 of these, the tumors were of mammary origin and grew progressively until death. One mouse even developed a new tumor during the 4th week of therapy. A tumor in a virgin ZBC mouse also grew progressively until death. In 2 other Z mice the tumors disappeared completely. One of these mice had what appeared to be a mammary tumor measuring 5 $\times$ 6 mm. at the start of therapy. The tumor regressed some and was sloughed off on the 10th day. Therapy was continued for a total of 63 days, and the animal was observed for an additional 42 days. There was no evidence of return of the tumor. The other mouse had a tumor near the center of the back of the thorax, which was probably not mammary in origin. This tumor measured 9 $\times$ 10 mm. at the beginning of therapy, regressed to 2 $\times$ 2 mm. in 2 weeks, and disappeared on the 16th day. Therapy was continued until death at 56 days, during which time the tumor did not return. In addition, 2 more Z females with mammary tumors showed beginning regression at 16 and 25 days, respectively, at which time they died, probably as a result of drug toxicity.

Discussion

5-Fluorouracil prevented growth of second- to fourth-generation transplants of a mammary adenocarcinoma but did not eradicate the malignancy. Tumors grew after cessation of therapy or when transplanted into untreated mice.

5-Fluorouracil seemed to be the cause of complete regression of spontaneous tumors in 2 of 28 mice and of beginning regression in another 2. Since biopsies of tumors were not made, it is impossible to be sure that these were malignant tumors. However, 1 that regressed completely

TABLE 3.—*Effect of 5-fluorouracil on the transplantability of tumors**

Donor mice			
Number of mice	Diameter of tumor in mm.		Change body weight (*gm.*)
	Start	2 Weeks	
Treated 7†.....	1.4 × 1.6	1.1 × 1.1	−1.2
Controls 10....	1.5 × 1.8	4.2 × 5.6	+1.8

Recipient mice						
Tumors	Number of donors	Number of recipients‡	Tumor palpable (*weeks*)	Diameter of tumor in mm.§		
				4 Weeks	8 Weeks	12 Weeks
Treated.....	7	7	3.0	2.0 × 2.2 (7)	7.6 × 8.9 (7)	11.1 × 12.7 (6)
Controls.....	10	48	1.8	4.2 × 5.8 (48)	9.2 × 11.0 (46)	14.7 × 18.8 (35)

*Mice were treated with 25 mg. per kg. of 5-fluorouracil for 9 days, then 20 mg. per kg. for 5 days, at which time tumors were transplanted to recipient mice.

†Thirteen of 20 treated mice died.

‡Treated tumors were transplanted into 9 recipients but failed to become established in 2 mice. Control tumors failed to become established in 2 of 50 mice. Because of the small size of treated tumors the whole tumor was transplanted in 5 mice and half a tumor in 4 mice.

§Numbers in parentheses are number of mice alive at that time.

TABLE 4.—*Growth of tumors after cessation of therapy**

Number of mice	Diameter of tumors in mm.					
	During therapy		Weeks after therapy:			
	Start	End	2	4	8	12
Treated 6.....	1.7 × 1.8	0.7 × 0.7	1.7 × 1.8 (6)†	5.1 × 6.7 (6)	12.4 × 16.8 (5)	19.0 × 23.0 (4)
Controls 10.....	1.6 × 1.7	5.8 × 6.0	10.0 × 11.6 (10)	13.1 × 16.7 (10)	19.1 × 22.7 (6)	23.0 × 28.0 (2)

*Mice were treated with 25 mg. per kg. of 5-fluorouracil for 2 weeks, then kept for 12 weeks after therapy. Three of 9 treated mice died during therapy.
†Numbers in parentheses are number of mice alive at that time.

and the 2 that regressed partially looked and felt like other spontaneous mammary adenocarcinomas in our mice. The other tumor which regressed completely was probably not of mammary origin but felt like a solid tumor.

It is apparent from the mortality figures presented in table 1 that the margin of safety between effective doses of 5-fluorouracil and lethal doses is small. This resembles the clinical findings of Curreri *et al.* (*5*), who noted tumor regression only in patients showing severe drug toxicity.

As mentioned above, 5-fluorouracil has been shown to have considerable activity against several types of transplanted tumors and microorganisms. Heidelberger *et al.* (*2*) have shown that the drug inhibits the conversion of formate to thymine. This interference with the synthesis of nucleic acids may constitute its mechanism of action on the growth of tumors and microorganisms. A side effect noted in the present work was the gradual development of a scaliness of the skin in animals on prolonged therapy. This may also be related to the antimetabolic activity of 5-fluorouracil.

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Further Clinical Studies With 5-Fluorouracil [1,2,3]

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Summary

Carcinoma of the breast, ovary, large bowel and rectum, and hepatoma appeared sensitive to 5-fluorouracil. While several favorable responses were seen with 5-fluorouracil in carcinoma of the cervix, anaplastic carcinoma (primary unknown), transitional cell carcinoma of the nasopharynx, reticulum-cell sarcoma, and synoviosarcoma, further clinical trial is needed to evaluate effectiveness in these lesions. There was strong evidence that 5-fluorouracil had no value in carcinoma of the lung, hypernephroma, carcinoma of the stomach and the pancreas, and malignant melanoma. In our opinion, the dosage schedule influenced the clinical response of the lesion to this agent. The most promising dosage regimen was the administration of 15 mg. per kg. per day for 5 days, followed by half doses given on alternate days until the appearance of early toxicity. So-called maintenance dosage schedule was not only ineffective, but may have been deleterious to the patient. It was necessary to repeat courses of treatment at approximately monthly intervals. The same dosage schedule of 5-fluorouracil could not be repeated, as a rule, for more than several months, and perhaps the dose should have been slightly reduced. 5-Fluorouracil is a toxic drug and should be given with extreme caution. No patient has been cured by this drug.—**J. Nat. Cancer Inst. 22: 497–507, 1959.**

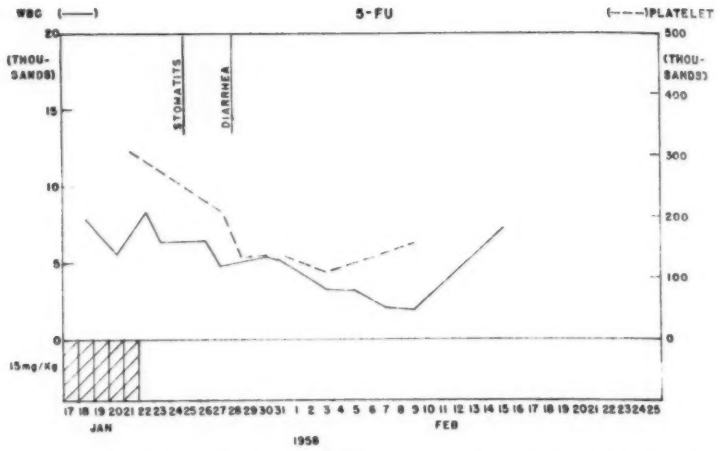
5-Fluorouracil (5-FU), developed jointly at the University of Wisconsin and Hoffmann-La Roche, Inc. (*1*, *2*), was administered over a period of 21 months to 190 patients with far-advanced metastatic neoplastic disease. The first 54 patients received less than 15 mg. per kg. per day for 5 days, and they represented the phase of our study concerned with the early determination of toxicity. Of the remaining 136 patients, 107 received a therapeutic trial of this compound for 2 months or longer.

The toxic manifestations and a preliminary report of the therapeutic effect of 5-fluorouracil were reported earlier (*3*). This report deals with added experience obtained with this agent.

[1] Received for publication September 5, 1958.

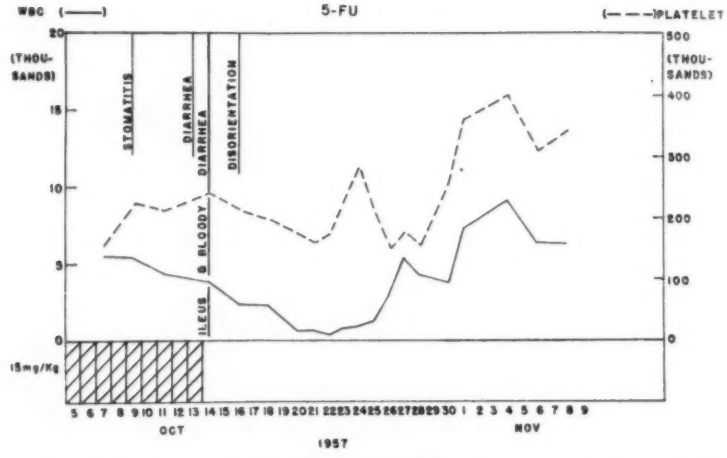
[2] This investigation was supported in part by the following grants-in-aid: Cy-2859, National Cancer Institute, Public Health Service; American Cancer Society, Inc., Wisconsin Division; Milwaukee Braves Baseball Club.

[3] 5-Fluorouracil generously supplied by Hoffman-La Roche, Inc., Nutley, N.J.



TEXT-FIGURE 1.—A typical course of 5-FU, 15 mg. per kg. per day for 5 days, demonstrating the onset of stomatitis, diarrhea, and subsequent bone-marrow depression.

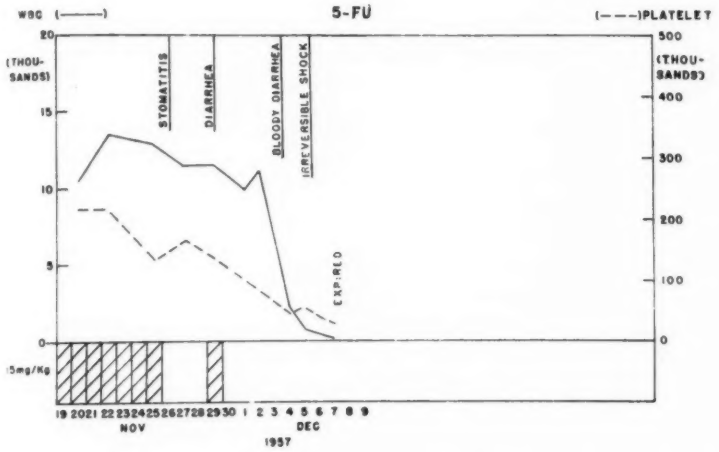
In the course of this study, tumor regression was observed only in patients treated to a toxic level, and an effort was made, therefore, to attain minimal to moderate toxicity in all instances. This level of toxicity was characterized not only by nausea and vomiting but more significantly by stomatitis, diarrhea, and subsequent bone-marrow depression as shown in text-figure 1. When toxicity progressed to a



TEXT-FIGURE 2.—A case of severe toxicity resulting from 5-FU therapy, 15 mg. per kg. per day for 9 days, demonstrating the appearance of stomatitis, diarrhea, subsequent ileus and bloody diarrhea, disorientation, severe bone-marrow depression, and ultimate recovery. (*Note:* A course of 5-FU therapy is now terminated with the appearance of stomatitis or moderate diarrhea.)

more extreme degree, ileus, bloody diarrhea, central nervous system depression, and pneumonia became evident (text-fig. 2).

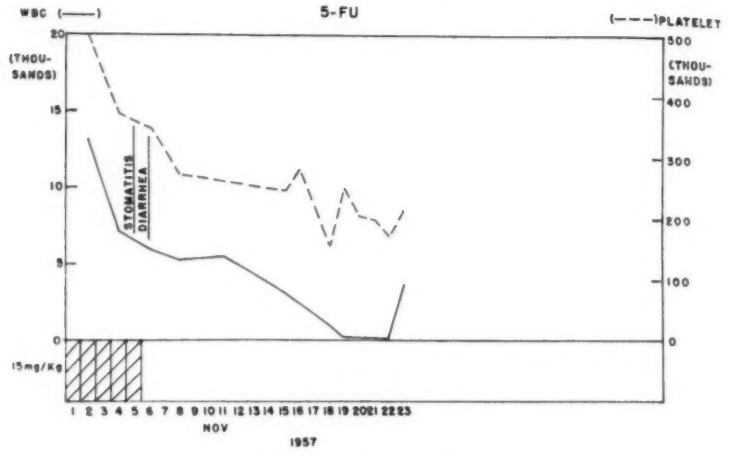
Seven deaths in our series were attributed to the toxicity caused by 5-fluorouracil. The terminal clinical picture was typically one of extensive bilateral pneumonia, irreversible shock, and hemorrhage from the gastrointestinal tract and tracheobronchial tree. Necropsy studies in each instance showed a characteristic ulcerative and hemorrhagic ileitis and jejunitis, and several cases revealed a complete loss of mucous membrane (figs. 1 and 2). In addition, there was bilateral pneumonia and hemorrhagic ulceration of the large bowel with agranulocytosis of the bone marrow (text-fig. 3).



TEXT-FIGURE 3.—A case of severe toxicity following 5-FU therapy, 15 mg. per kg. per day for 7 days, followed by the same dose 4 days later, demonstrating severe bone-marrow depression.

5-Fluorouracil was poorly tolerated by patients who previously had received alkylating agents or heavy doses of radiation to large areas of bone marrow, particularly to the pelvis. In either of these circumstances a precipitous drop in white blood cell counts and platelet counts often occurred following the administration of the usual course of this compound (text-fig. 4).

Evaluation of the results requires defining the terms "improved" and "unimproved." Patients were classified as improved when all the following criteria were met: 1) measurable reduction in tumor size; 2) symptomatic improvement; 3) definite improvement in performance status as shown by return to partial or complete former physical activities; 4) leveling off or reversal of downward weight curve; 5) maintenance of these changes for a minimum of 2 months. Patients were considered unimproved when no response to 5-FU was noted.



TEXT-FIGURE 4.—Severe decline in WBC count following administration of 5-FU therapy, 15 mg. per kg. per day for 5 days, in a patient who had received pelvic radiation 5 years earlier.

Clinical Response to 5-FU in Various Types of Malignant Lesions

On the basis of these definitions various malignant tumors were placed in 4 groups according to their therapeutic response to 5-fluorouracil. The first group shown in table 1 included those malignant lesions where objective improvement might be anticipated. The most dramatic responses occurred in carcinoma of the breast and in primary hepatomas. Regression of tumors and general well-being were maintained up to 13 months. In only 2 cases did objective improvement occur for as little as 2 months.

TABLE 1.—*Malignant lesions in which objective improvement might be anticipated*

Lesion	Improved	Unimproved
Carcinoma of breast	15	2
Carcinoma of large bowel and rectum	8	8
Carcinoma of ovary	2	1
Hepatoma	2	0
Total	27	11

In contradistinction to the first category, carcinoma of the lung, stomach, pancreas, as well as hypernephroma and malignant melanoma, utterly failed to respond to 5-fluorouracil (table 2).

In a third group, a number of neoplasms was included whose response to this compound was not consistent or in which there was but a single instance of success (table 3). The management of carcinoma of the cervix was most disturbing. Six of our 7 patients had received the usual extensive radiotherapy to the pelvis prior to the use of 5-fluoro-

TABLE 2.—*Malignant lesions not responding to 5-FU*

Lesion	Improved	Unimproved
Carcinoma of lung	0	16
Hypernephroma	0	6
Carcinoma of stomach	0	5
Carcinoma of pancreas	0	4
Malignant melanoma	1	5
Total	1	36

TABLE 3.—*Malignant lesions which may respond to 5-FU*

Lesion	Improved	Unimproved
Carcinoma of cervix	3	4
Carcinoma, primary unknown	2	3
Reticulum-cell sarcoma	1	1
Transitional cell carcinoma of nasopharynx	1	0
Synoviosarcoma	1	0
Total	8	8

TABLE 4.—*Individual cases of malignant lesions failing to respond to 5-FU*

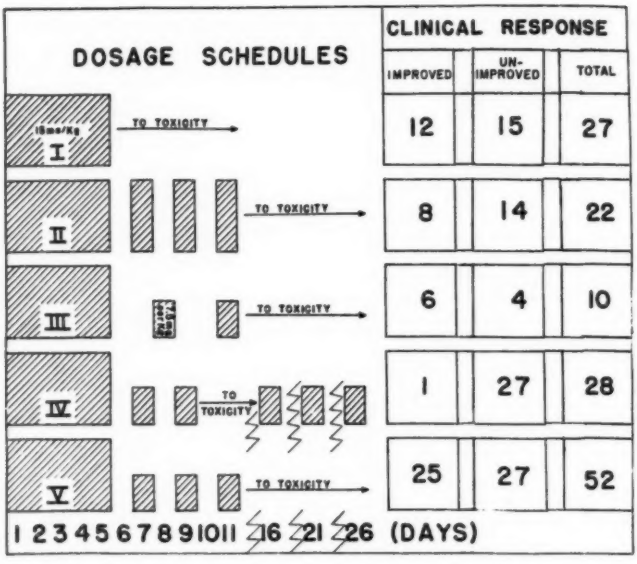
Lesion	Improved	Unimproved
Leiomyosarcoma	0	1
Osteogenic sarcoma	0	2
Fibrosarcoma	0	1
Liposarcoma	0	1
Glioblastoma multiforme	0	1
Carcinoma of the:		
Thyroid	0	1
Mandible	0	1
Face	0	1
Embryonal cell of testis	0	1
Tonsil	0	1
Larynx	0	1
Total	0	12

uracil. Five of them developed extreme toxicity, with death occurring in 3. Our present policy is to accept stage-IV cases of carcinoma of the cervix without previous radiotherapy.

In a fourth group, single cases of various neoplasms failed to respond to 5-FU (table 4).

Clinical Results as Influenced by Various Dosage Schedules

In an effort to develop a regimen which would be therapeutically effective and still not too toxic or hazardous, 5 dosage schedules were studied (text-fig. 5). The first consisted of daily injections of 15 mg. per kg. per day until toxicity appeared. Twenty-seven patients were treated with this regimen and 12 showed improvement, but 15 were unimproved.

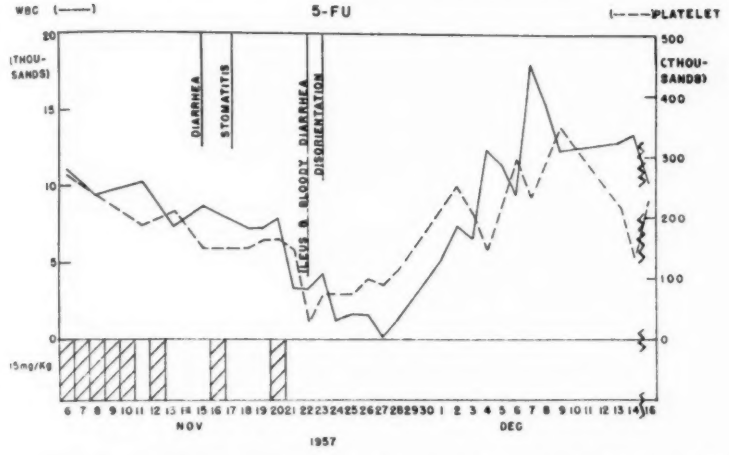


TEXT-FIGURE 5.—The clinical response as influenced by the 5 dosage schedules employed in the study.

In view of the severe toxicity which occurred in several patients (text-fig. 2), a second schedule was established in which 15 mg. per kg. per day was given for 5 days. The dose was then repeated every 2 or 3 days until early toxicity was also noted in this group. Of the 22 patients treated in this manner, 8 improved and 14 were unimproved.

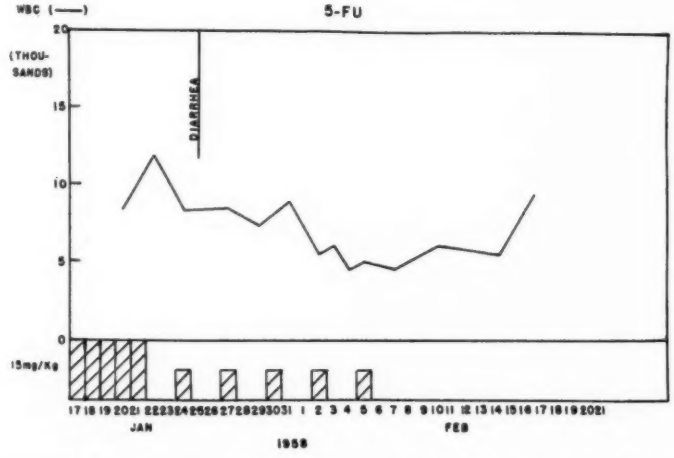
Again, with this schedule, a number of instances of severe toxicity occurred (text-fig. 6) and consequently a third schedule was adopted which provided the same basic 15 mg. per kg. per day for 5 days but which was followed by 7.5 mg. per kg. every 3d day. In this study stomatitis appeared in only 2 patients while it was a common occurrence in the first 2 groups. Only 10 patients were treated with this regimen, and 6 improved while 4 were unimproved. Severe toxicity was not observed on this schedule but hospitalization was unduly prolonged. A graph illustrating this schedule is shown in text-figure 7.

In an attempt to decrease the severe toxicity as well as to reduce the hospitalization period to a minimum, a fourth schedule was developed which provided 15 mg. per kg. per day for 5 days, followed by a half dose on alternate days to the point of toxicity. Patients were then discharged from the hospital and maintained on the half dose every 5th day on an outpatient basis over a prolonged period. Twenty-eight patients were placed on this regimen and only 1 improved while 27 were unimproved. In a number of instances it was felt that the neoplastic process was accelerated in this course of therapy. Text-figure 8 illustrates the course of a patient treated by this regimen.

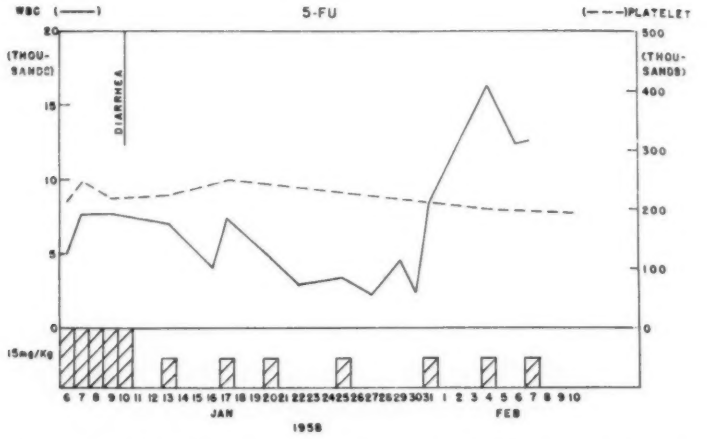


TEXT-FIGURE 6.—A case of severe toxicity with ultimate recovery in a patient treated with 5-FU, 15 mg. per kg. per day for 5 days, followed by this dose at 2- to 4-day intervals.

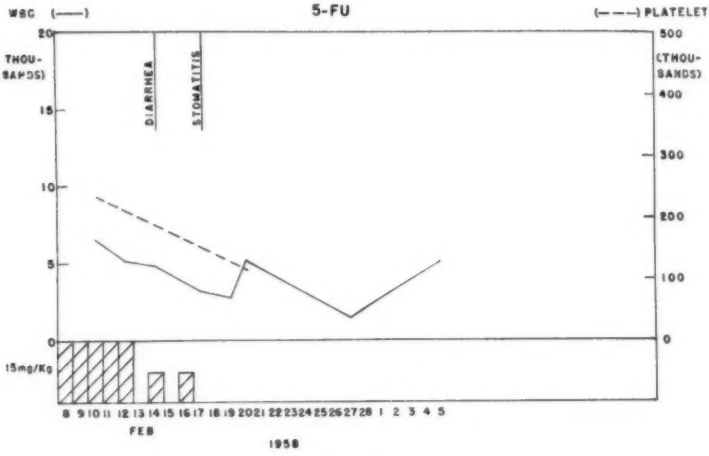
The fifth schedule, which we presently employ, consisted of administering 15 mg. per kg. per day for 5 days intravenously, followed by a half dose on alternate days until early toxicity appeared. Since bone-marrow depression occurred between the 14th and 20th day, this could not be considered as an early toxic sign. Of 52 patients thus treated, 25 improved and 27 were unimproved. This course is illustrated in text-figure 9.



TEXT-FIGURE 7.—The course of a patient receiving 5-FU, 15 mg. per kg. per day for 5 days, followed by 7.5 mg. per kg. every 3d day. (*Note:* No toxicity developed and hospitalization was prolonged to 3 weeks.)



TEXT-FIGURE 8.—The course of a patient receiving 5-FU, 15 mg. per kg. per day for 5 days, followed by the half dose every 3 to 6 days over a prolonged period.



TEXT-FIGURE 9.—The course of a patient receiving 5-FU, 15 mg. per kg. per day for 5 days, followed by the half dose on alternate days until toxicity appears. (*Note:* The course is terminated with the onset of stomatitis.) WBC count reached the lowest level 10 days after onset of stomatitis.

Each course of treatment was repeated at monthly intervals, because in patients with measurable lesions it was found that progression ensued if a period of 5 or more weeks were permitted to elapse between courses. A word of caution is needed regarding the repeating of the initial dosage schedule over several months. Often patients manifested increased toxicity with later courses of therapy and required a reduction of one-half or one daily dose, depending upon the toxicity elicited during the last

course. This reduction in dosage did not alter the remission produced in the lesion.

At the time of our previous report, no instance of drug resistance was encountered. Since then, 3 patients became resistant. Two others became sensitive to the drug and an adequate dose could not be administered. These patients presented very severe bone-marrow depression when they received 4 or less doses of the agent.

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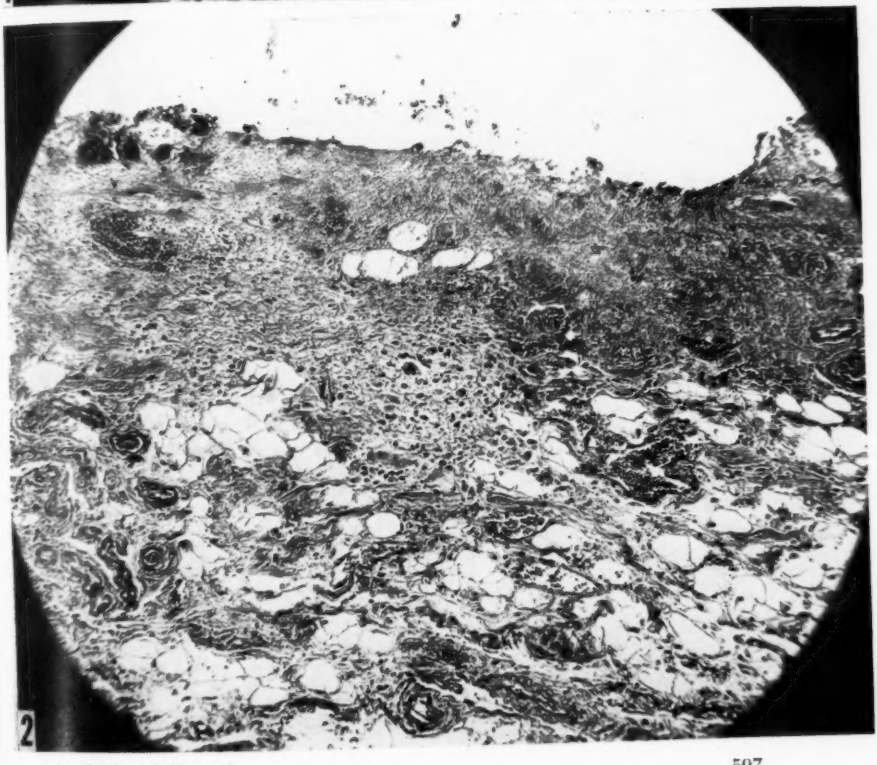
PLATE 49

FIGURE 1.—Ulcerative esophagitis showing loss of mucosa.

FIGURE 2.—Ulcerative ileitis showing loss of mucosa.



493212—59——7

A Transplantable Plasma-Cell Leukemia in Mice Associated With the Production of β-Paraprotein [1,2]

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Summary

The first 8 transfer generations of a spontaneous plasma-cell leukemia originating in a 27-month-old (CBA × DBA/2)F_1 mouse are described. Percentage of takes was 57 to 100. Survival time in the first passage was 148 to 437 days, gradually shortened to 55 to 193 days in the seventh passage. Total serum protein was normal. Paper electrophoresis showed a marked increase of the β-globulin fraction. Immunoelectrophoresis revealed that the paraprotein was a γ-protein of β-mobility; a $\beta_{2\text{-III}}$-fraction of increased concentration and mobility was found, probably due to a reaction of the host toward the implanted neoplasm. In starch-gel electrophoresis the paraprotein migrated more slowly than the normal β-globulin, and the $\beta_{2\text{-III}}$-fraction migrated faster. From the sixth passage the amount of paraprotein in serum decreased. Blood changes consisted in relative granulocytosis, in the last days of survival associated with the outburst of plasma cells. The outstanding macroscopic lesions were enlargement of the thymus, liver, spleen, and lymph nodes. Microscopic changes were typical leukemic infiltrations in all predilection sites of leukemia. Differentiation grade of the plasma cells in the spontaneous case and in transplanted mice of the first 2 passages was grade II-III; in the next 5 passages, grade III; and in the 8th transfer, grade III-IV. The relative granulocytosis developed simultaneously with the early manifestations of the leukemic lesions in the organs; paraprotein was released into the blood when the leukemic lesions had developed to a moderate degree, increasing in amount with further development of the leukemic process. Plasma cells did not enter the blood stream until the leukemic infiltrations had become extremely marked. No paraprotein was observed in the urine of leukemic mice.—**J. Nat. Cancer Inst. 22: 509–541, 1959.**

Since 1951, when the occurrence of plasma-cell leukemia in mice of the ST strain was first described (*1*), several reports on plasma-cell tumors in mice have been published (*2–8*).

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[2] These investigations were supported by grants from The Leukemia Society, Inc., New York, N.Y U.S.A., and Civilingeniør H. Jacob Nielsens Legat, Dansk Ingeniørforening, Denmark.
[3] Holding a scholarship from Købmand i Odense, Johann og Hanne Weimann, f. Seedorffs Legat, Copenhagen, Denmark.

493212—59——8

The plasma-cell leukemias observed by Rask-Nielsen and Gormsen (*8*) were found in DBA, DBA/2, C3H, C57BL, CBA, (DBA × C57BL)F_1, (C3H × C57BL)F_1, and (DBA/2 × CBA)F_1 hybrid mice; widespread leukemic lesions were seen in all predilection sites of leukemia. No blood examinations and no attempts at transplantation were made at that time.

The plasma-cell neoplasm described by Bichel (*2*) was a subcutaneous plasmocytoma of an AK mouse. When transplanted subcutaneously, local growths developed, with scarce dissemination to other tissues; relative granulocytosis and, in a few cases, some plasma cells were found in the blood. No serological examinations were made.

The neoplasms observed by Dunn (*3*, *4*, p. 1367, *5*) and by Potter and Law (*6*, *7*) were ileocecal tumors in C3H mice. On transplantation, only one of the plasmocytomas took; it grew locally with only slight spreading to other organs. No abnormal serum-protein changes and no leukemic blood changes were found.

In 1957, Potter, Fahey, and Pilgrim (*9*) described another ileocecal C3H plasmocytoma, which, when transplanted, was associated with the development of osteolytic bone lesions and with the occurrence of an abnormal serum-protein fraction in the γ-globulin range. No blood changes were mentioned.

Shortly after the report by Potter *et al.*, Rask-Nielsen, Clausen, and Gormsen (*10–12*) gave a preliminary report on two transplantable plasma-cell leukemias in mice, which gave the same type of paraprotein patterns in serum as that associated with human myelomas and plasma-cell leukemias. One line (transplantation line 20) was constantly associated with a paraprotein fraction in the β-globulin region, as estimated from paper electrophoresis. The other line (transplantation line 36) was characterized by the occurrence of an abnormal protein fraction in the area between the β- and γ-regions, termed M-paraprotein. In both lines plasma cells in various numbers were observed in the blood, particularly premortally.

Recently, Clausen *et al.* (*13*) have shown in immunoelectrophoretic studies that the paper electrophoretically demonstrated M-paraprotein of transplantation line 36 immunologically consists of an abnormal γ-protein fraction and that the β-paraprotein of line 20 immunologically is a γ-protein of β-mobility.

Both lines of leukemia originated in (CBA × DBA/2)F_1 hybrid mice. Rask-Nielsen (*14*) observed that the incidence of leukemia and reticulum-cell sarcoma in old mice of this genetic constitution was about 50 percent. These neoplasias developed in 96 of 213 mice that were more than 18 months of age; the majority of the leukemias were of the plasma-cell variety.

So far, a total of more than 50 plasma-cell leukemias and reticulum-cell sarcomas besides some lymphatic leukemias have been successfully transplanted. At least 9 plasma-cell transplantation lines have been found associated with the development of paraprotein, whereas other trans-

plantation lines of plasma-cell leukemia as well as the lines of reticulum-cell sarcoma and lymphatic leukemia have not shown such serological abnormalities.

Often the survival time of the first transfer generation was 12 to 15 months.

Investigations of most of these transplantation lines are still in progress and will be published in separate communications starting with the present paper, which gives a more detailed report on the serological, hematological, and histological findings observed in mice of the first 8 transfer generations of the previously mentioned β-paraprotein-developing plasma-cell leukemia, transplantation line 20.

Materials and Methods

Animal material.—(CBA $\times$ DBA/2)F_1 hybrid mice were used for all experiments. The hybrids and the parent strains were all bred in this laboratory; the parent strains were bred by brother $\times$ sister mating. The mice were kept in metal cages, 5 or 6 in each cage, and were fed whole wheat and pellets, which were prepared for egg-laying poultry and declared to contain 22 percent protein, 3 percent fat, 40 percent carbohydrate, plus adequate quantities of vitamins and mineral salts. The supply of drinking water was unrestricted.

Technique of transplantation.—Leukemic tissue (subcutaneous growths, lymph nodes, spleen, or liver) was minced, and drawn into a syringe. Using a needle with approximately 1.5 mm. internal diameter, 0.05 ml. was injected subcutaneously into the left flank near the axilla.

Technique of blood sampling.—Blood was obtained from a tail vein or, if the mouse had to be killed, from the axillary vessels. For white-blood-cell counting, 0.01 ml. of blood was mixed with 0.2 ml. methyl-violet-acetic acid, and counted in a Bürker-Türk counting chamber. Blood smears and imprint preparations of organs were fixed in methanol and stained with Giemsa's solution diluted 1 : 10 with buffered water, *p*H 6.5.

Serum was obtained in the following way: No more than a 6 to 7 cm. column of blood was drawn up into a glass capillary tube, 1.5 to 1.8 mm. in diameter and 10 cm. in length; when the blood had clotted, the free end of the tube was sealed by heating. The tube was centrifuged until the blood cells and serum had completely separated; then the capillary tube was broken just above the surface of the red cells and the serum drawn directly into a capillary pipette for immediate use, or stored in the sealed capillary tube at $-20°$ C.

Quantitative determination of serum protein.—The method used was that of Lowry *et al.* (*15*) for colorimetric determination of protein in cerebrospinal and other biological fluids. This method is based on a reaction between protein and the phenol reagent of Folin-Ciocalteau in the presence of $CuSO_4$. A solution of 13 mg. percent tyrosine in 0.1 N HCl was used as a reference standard. This standard corresponds to 50 mg. percent protein (*16*).

Ten μl. of serum was diluted 1:100 with 0.1 N NaOH; two separate

analyses were made on 0.1 ml. of this dilution. The determinations were made in a Beckman spectrophotometer at 750 mμ.

Technique of paper electrophoresis.—Paper electrophoreses were run on 10 μl. of serum in an apparatus of the type designed by Laurell *et al.* (*17*). Their technique was followed, with the modification that an acetate-Veronal-calcium buffer, *p*H 8.6, ionic strength 0.05 was used. In later experiments the distilled water of the Veronal buffer was replaced by tap water and the addition of calcium lactate was omitted. Whatman filter paper #1 was used. The strips were run for 17 to 18 hours at 100 volts. Under these experimental conditions the fastest migrating protein fraction moved about 9 cm. After the strips were dried at 110° C., they were stained with bromphenol blue, rinsed 3 times in 0.5 percent acetic acid, and allowed to dry in the air.

The patterns of the strips were evaluated by transmission densitometry in an ELL scanner, and the areas of the peaks, measured planimetrically, were used as a basis for estimating the relative percentages of the various protein fractions.

Technique of immunoelectrophoresis.—The method used was initially developed by Grabar and Williams (*18*). It was modified to a micromethod by Scheidegger (*19*), and further modified by Heremans (*20*). In principle this method is based on the precipitation lines of the electrophoretically separated protein fractions that will develop when antiserum, prepared by immunization of an animal of a different species, is allowed to diffuse toward these protein fractions.

The immunoelectrophoretic procedure and pattern in our normal (CBA × DBA/2)F_1 hybrid mice have been described recently in a separate paper (*21*).

Technique of zone electrophoresis in starch gels.—The technique employed was developed and used by Smithies (*22*), and later by Galatius-Jensen (*23*). In the prepared starch-gel medium the migration of some protein molecules seems not only to be a function of their electrical charge but also to be related to the size of the molecules, probably because the starch gel functions as a filter, with a pore size about the same diameter as the serum-protein molecules. Twenty-five μl. of serum was used for each determination.

The total amount of blood required for these analyses is 50 to 100 μl. In addition one drop was used for a blood smear. This small volume could be drawn from the same mouse at intervals of only 2 to 3 days.

Macroscopic and microscopic examinations.—All mice were autopsied. If leukemic lesions were observed or suspected, the lung, liver, spleen, kidney, lymph nodes, and subcutaneous growth, when present, were routinely removed, fixed in formalin, and stained with hematoxylin and eosin. Other organs, if pathologically changed, were also examined.

The following special staining methods were employed: van Gieson, Unna Pappenheim, periodic acid-Schiff (PAS), and reticulin-staining (Wilder).

Often imprint preparations of infiltrated organs were used for supplementary elucidation of the findings observed in the histological sections.

Plasma-cell leukemias have been classified in 4 grades of differentiation according to the degree of maturity of the plasma cells. The criteria for this classification were as follows: In grade I the majority of the plasma cells were typical mature Marchalko plasma cells; in grade IV many of the cells were undoubtedly reticulum cells showing transition into plasma cells, the histological picture resembling reticulosarcoma. Grades II and III were intermediary varieties. Even transitions between the various grades have been observed. For further description and for illustrations of the differentiation grades, the reader is referred to our earlier papers (*1, 8*).

Experiments

Serological and Hematological Examinations in Normal Mice

Blood from a tail vein of 10 normal (CBA × DBA/2)F_1 hybrid mice, 5 to 6 months of age, was examined for comparison. The results were as follows:

Serological findings.—Paper electrophoretic determinations:

Albumin	47.8±2.3 percent	(minimum	45 percent,	maximum	55 percent).
α_1-Globulin	6.7±1.8 percent	"	3.8 percent,	"	9.5 percent).
α_2-Globulin	16.6±2.5 percent	"	13 percent,	"	20 percent).
β-Globulin	18.1±1.5 percent	"	16 percent,	"	21 percent).
γ-Globulin	11.0±2.3 percent	"	8 percent,	"	14 percent).

Total amount of protein:

6.54±0.26 gm. percent (minimum 6.1 gm. percent, maximum 6.7 gm. percent).

Blood picture.—Number of white blood cells:

6,713±3,080 per mm.^{3} (minimum 3,640, maximum 12,530).

Differential counts of blood smears:

Lymphocytes	78±9.5 percent	(minimum	75 percent,	maximum	87 percent).
Granulocytes	20±2.4 percent	"	12 percent,	"	24 percent).
Monocytes	2±1.2 percent	"	0.5 percent,	"	4 percent).

Plasma-cell Leukemia, Transplantation Line 20

Spontaneous case.—This line of leukemia originated in a 27-month-old female (CBA × DBA/2)F_1 hybrid mouse that, at the age of 1 month, had had thymic tissue from a DBA/2 mouse implanted subcutaneously.

Evidence that the progenitor cells of this leukemia were of F_1 hybrid origin and not DBA/2 cells was given by a histocompatibility test, in which no takes resulted from transplantation into DBA/2 mice. Furthermore since the experiment concerning implantation of DBA/2 thymic tissue showed a negative result (*14*), the leukemia must be considered to have developed spontaneously.

Examination of blood from a tail vein at 7 weeks, 3 weeks, and 1 week before the mouse was killed, and at the date of death, revealed a normal blood picture showing 5,500 to 11,500 white blood cells per mm.^{3}, of which 70 to 75 percent were lymphocytes, 20 to 25 percent were granulocytes, and 2 to 8 percent were monocytes.

Paper electrophoresis of serum performed 2 weeks before the mouse was killed showed that albumin made up 49 percent, α_1-globulin 10 percent, α_2-globulin 7 percent, β-globulin 29 percent, and γ-globulin 5 percent.[4] Since the β-globulin fraction in our normal (CBA $\times$ DBA/2)F_1 mice makes up only 16 to 21 percent, this means a definite augmentation of the β-fraction.

The mouse was not particularly sick when killed, but the peripheral lymph nodes were pea-sized. At autopsy this finding was verified; in addition, slight enlargement of the thymus and spleen was observed and the pancreatic lymph node had developed into a tumor, 10 by 10 by 8 mm.

Microscopic examination revealed heavy infiltration of the spleen and lymph nodes and moderate infiltration of the thymus, lung, liver, kidney, and perirenal tissue. The leukemic infiltrations consisted of plasma cells, differentiation grade II to III according to the criteria previously mentioned (fig. 2). Furthermore, in the bone marrow of a lumbar spine, one single, small, circumscribed nodule of plasma cells was observed (fig. 3).

Results of Transplantation

Incidence of takes.—The leukemia has so far been carried through eight transfer generations. As indicated in table 1 there were 81 percent takes—the variation being from 57 to 100 for all 50 mice of the eight passages.

Survival time.—From table 1 it is evident that the survival time within each transfer generation has varied to a great extent; in the first passage, from 148 to 437 days; in the third passage, from 112 to 250 days; and in the seventh passage, from 55 to 193 days. Besides this variation, it may be seen that the survival time, particularly of the mice succumbing earliest, within each passage has shortened in the later passages.

Total serum protein.—The amount of total serum protein was within normal limits (6.1 to 6.7 gm. %). In some mice there was a decrease to about 4.5 gm. percent as the lowest concentration during the last days of survival, with the same relative quantitative distribution of the fractions as seen in leukemic mice with a normal amount of protein.

Serological Changes Seen in Paper Electrophoresis

Figure 4b shows the typical changes observed in paper electrophoretic strips of serum from a mouse of the fourth transfer generation (No. 7411). A pronounced increase of the β-globulin fraction is noted as compared to this fraction in a normal mouse (fig. 4a). The β-fraction of the pathological serum is 43 percent of 6.4 gm. percent of total serum protein, or 2.7 gm. percent. In normal serum this fraction is 18 percent of 6.5 gm. percent, or 1.2 gm. percent. Simultaneously, the albumin fraction shows a decrease from 48 percent, or 3.1 gm. percent, in the normal serum to 36 percent, or 2.3 gm. percent, in the pathological serum.

[4] This electrophoretic determination was undertaken at the Central Laboratory of Frederiksberg Hospital, Copenhagen, through the courtesy of the head of the laboratory, A. Levin Nielsen, M.D., to whom our sincere thanks are due.

TABLE 1.—*Incidence of takes, survival time of leukemic mice, frequency of macroscopic and microscopic lesions in eight transfer generations of plasma-cell leukemia, transplantation line 20*

Passage of transplantation	Number of takes	Percentage of takes	Survival time of leukemic mice (*days*)	Number of leukemic mice	Number of mice with macroscopic lesions in:					Number of mice examined microscopically	Number of mice with microscopic lesions in:				
					Thymus	Liver	Spleen	Perirenal tissue	Lymph nodes		Lung	Liver	Spleen	Kidney	Lymph nodes
1	3/4	75	148, 421, 437	3	1	1	3	1	3	3	2	3	3	2	3
2	6/6	100	99, 100, 102, 105, 105, 118	6	4	1	4	6	5	2	1	2	2	2	2
3	5/6	83	112, 155, 176, 176, 250	5	5	1	4	5	5	5	5	5	5	2	5
4	4/7	57	133, 114, 125, 227	4	3	4	4	3	4	3	3	3	3	3	3
5	4/4	100	70, 96, 97, 156	4	4	3	4	4	4	2	1	2	2	0	2
6	7/9	78	42, 55, 72, 74, 89, 93, 122	7	6	4	7	7	7	3	3	3	3	2	3
7	7/9	78	55, 67, 80, 136, 141, 143, 193	7	7	7	7	7	7	4	4	4	4	3	4
8	4/4	100	38, 40, 44, 62	4	4	4	4	4	4	2	2	2	2	2	2
Total	40/49	81		40	34	25	37	37	39	24	21	24	24	16	24
Percent					85	62	92	92	97		88	100	100	66	100

This large increase in the β-fraction was observed in the mice from the first five transfer generations; then a decrease of the β-fraction started, and in the seventh and eighth passages it amounted to 25 to 30 percent of total protein. The other fractions remained unchanged.

The pathologically increased β-fractions are rich in mucopolysaccharides (*10*), thus labeling the paraprotein as a glycoprotein.

Serological Changes Seen in Immunoelectrophoresis

As recently reported (*13*), immunoelectrophoretic studies were carried out on serums from mice of this line of leukemia.

It may be recalled that serum from our normal (CBA × DBA/2)F_1 hybrid mice has been shown (*21*), in immunoelectrophoresis, to develop one long γ-globulin bow extending far into the β-region, and three β-globulin fractions, termed β_1-, β_2-, and β_3-, of which β_3- is the slowest and β_1- the fastest migrating part. β_3- was seen to consist of three bows, called $\beta_{3\text{-I}}$-, $\beta_{3\text{-II}}$-, etc.; β_2- was fractionated into four bows, termed $\beta_{2\text{-I}}$-, $\beta_{2\text{-II}}$-, etc.; finally only one β_1-fraction was found (fig. 5b).

An immunoelectrophoresis of serums from leukemic mice of the present line of leukemia (fig. 5c) demonstrated that the faster part of the γ-bow, that is, the portion extending into the β-region, was deformed, with a deflection toward the antibody reservoir, and revealed an increased intensity and sharpness; furthermore, the γ-bow could be followed much farther into the β-zone than normally. These abnormalities are all indicative of an excess of γ-paraprotein located in the β-region.

In addition, the $\beta_{2\text{-III}}$-fraction showed an increased concentration and mobility, and the $\beta_{2\text{-I}}$-bow, a slight augmentation (*cf. 13*).

Serums from the first three passages were not examined. In serums from mice of the fourth, fifth, and sixth passages the abnormalities just described were seen; yet, in serum from the sixth passage diluted 1:16, the pathological γ-fraction was less marked than in earlier passages.

When paper electrophoretic determinations had shown that the amount of paraprotein had decreased in the seventh passage, an immunoelectrophoretic study of serum from a mouse of this transfer was undertaken. In undiluted serum it was observed that both ends of the γ-bow had developed more faintly, and particularly that the intensity and deformation of the faster part of this bow was less pronounced, indicating a decreased excess of paraprotein. The abnormalities of the $\beta_{2\text{-III}}$-fraction, on the other hand, remained unchanged.

The paraprotein manifesting itself as abnormalities of the γ-bow must no doubt be considered a product of the plasma cells; whether it is secreted by them or released when they die is undecided. At first, the cause of the changes in the $\beta_{2\text{-III}}$-fraction was unknown. Since recent immunoelectrophoretic studies have shown (*24*) that identical abnormalities of this bow develop in mice carrying transplanted mammary and pulmonary carcinomas and in mice following injection of crystalline human albumin, it seems safe to conclude that the changes observed in the $\beta_{2\text{-III}}$-bow of the leukemic serums are the result of a reaction of the host organism toward the pro-

tein of the implanted neoplasms. This view is well in accordance with the fact that the $\beta_{2\text{-III}}$-fraction remained unchanged irrespective of the decrease in paraprotein seen in the leukemic mice of the later passages.

Serological Changes Seen in Zone Electrophoresis in Starch Gel

Considering the serological abnormalities in immunoelectrophoresis just mentioned, we thought it appropriate to have our leukemic serums examined by starch-gel electrophoresis also.[5] Figure 6 shows a confrontation of starch-gel electrophoresis of normal serum and serum from 2 leukemic mice, No. 7411 from the fourth passage and No. 2182 from the sixth passage.

As evident from figure 6, a part of the α_2-globulin zone, termed $S_{\alpha 2}$- (*22*), migrates more slowly than the β-globulin fraction. The location of the other normal protein fractions is in accordance with the findings in paper electrophoresis, except that the normal γ-globulin fraction in starch gels moves in a retrograde direction. Since the slit into which the serum sample is inserted prior to the running of the electrophoresis breaks the starch-gel strip, the portion of the strip with the γ-fraction is disconnected from the main part of the strip. For this reason the retrograde part of the starch-gel strips are not included in figure 6.

In starch-gel electrophoreses of the leukemic serums, an abnormal zone of slightly slower mobility than the normal β-globulin fraction is seen; this zone stains much more intensely in the serum from No. 7411 (fig. 6c) than in serum from No. 2182 (fig. 6b).

An abnormal zone of identical intensity in the serums from both leukemic mice can be observed just in front of the normal β-fraction; probably a corresponding, very faintly developed, zone is found in the normal serum—the albumin fractions clearly are decreased in the leukemic serums.

Starch-gel electrophoreses of serums from other leukemic mice of the present line have shown, in principle, the same abnormalities as those just described. The slowest-moving abnormal fraction decreased in the later passages, whereas the fastest-migrating pathological zone showed some variation, but no tendency to decrease.

Most probably the abnormal zone between the $S_{\alpha 2}$- and the β-fraction represents the paraprotein. This assumption is supported by the fact that in starch-gel electrophoresis we have found human paraprotein of β-mobility in this same area, whereas human paraprotein of γ-mobility migrated in the retrograde direction. Furthermore, the higher intensity of this zone in serum from mouse No. 7411 compared to that of No. 2182 is in accordance with the difference in the amount of paraprotein observed in paper electrophoresis of these serums.

Whether the pathological fraction of faster velocity than the normal β-globulin zone corresponds to the increased $\beta_{2\text{-III}}$-fraction in immunoelectrophoresis, we do not know; but it seems a rather tempting assumption, since the $\beta_{2\text{-III}}$-fraction belonged to the faster-moving β-globulins.

[5] The starch-gel electrophoreses were run in the Serological Department of the University Institute of Legal Medicine through the courtesy of F. Galatius-Jensen, M.D.

The similar intensity of this abnormal fraction in starch gel as well as in immunoelectrophoresis of leukemic serums, independent of different amounts of paraprotein, points to the same conclusion. And finally the fact that no such abnormal zone could be observed in human myeloma serum supports this assumption.

Juxtaposition of the Results of Three Methods of Electrophoresis

In paper electrophoresis, the paraprotein characteristic of the present line of leukemia was located in the β-globulin fraction and consequently was assumed to be a β-globulin. From starch-gel electrophoresis, it is evident that the paraprotein in starch gel has a velocity discernible from that of normal β-globulin. And, finally, immunoelectrophoresis was able to reveal that the paraprotein was immunochemically a γ-protein of β-mobility. Thus the paraprotein differs from other protein fractions in physical and immunochemical properties, which has also been observed in investigations using other procedures (*25–28*).

In paper electrophoresis, the particular protein fraction produced by the host, as a reaction toward the implanted neoplasm, was intermingled with the normal β-globulin fraction and the paraprotein; it was first revealed in immunoelectrophoresis, and was probably also observed in starch-gel electrophoresis. The occurrence of this protein fraction would not have been detected without the employment of one of the latter methods, especially immunoelectrophoresis, which, furthermore, was the method of choice in demonstrating the γ-globulin origin of the paraprotein.

Blood Changes

During development of the leukemia, a relative and, now and then, a slight, absolute granulocytosis was observed, which in the last days or, in a few cases, a couple of weeks before the mice died, was followed by outflow of plasma cells (figs. 7 and 9). Up to 92,000 white blood cells per mm.^{3}, 95 percent of which were plasma cells, have been observed *sub finem*, but usually the number of white cells did not exceed 20,000 to 30,000, and often did not reach abnormally high values.

Table 2 shows development of the blood changes and the simultaneously performed electrophoretic examinations on serums from 2 mice of the fifth and eighth passages, respectively. It will be seen that the relative granulocytosis preceded the occurrence of paraprotein in the serum and that plasma cells did not enter the blood stream in larger numbers until late in the disease.

When granulocytosis had existed for some time, more immature myeloid cells were observed in the blood. These cells were larger than normal granulocytes and had slightly twisted, ring-shaped nuclei, which had rather coarsely distributed, darkly staining chromatin and a more or less basophilic cytoplasm. The cells were found in largest numbers, up to 20 to 25 percent, when the leukemia took a protracted course.

The granulocytosis is probably a reaction from the host against the implanted malignant growth without any special relation to the plasma-

TABLE 2.—*Correlation of hematological and serological findings, observed in 2 mice during development of plasma-cell leukemia, transplantation line 20*

Days after inoculation	Days before death	Number of white blood cells	Differential count:				Total serum protein (*gm. percent*)	Electrophoretic determination:				
			Lymphocytes	Granulocytes	Monocytes	Plasma cells		Albumin	α_1-Globulin	α_2-Globulin	β-Globulin	γ-Globulin
Mouse No. 7802, fifth passage												
96	51		71	25	4			51	5	12	19	13
110	27		29	61	10			43	6	17	21	13
136	11		12	84	2	2		33	6	17	33	11
Mouse No. 2438, eighth passage												
82	54	8500	38	58	4		6. 5	51	7	15	24	3
89	47	9500	33	55	12		6. 0	50	4	14	24	5
104	32	10800	30	59	11		6. 4	46	7	19	25	3
118	18	13400	20	66	10	4	5. 9	42	9	15	26	8
134	2	6100	11	71	3	15	5. 8	48	5	18	25	4
136	0	20300	3	13	2	84	5. 2	47	5	15	27	6
Ten normal mice	Mean	6713	78	20	2		6. 54	48	6	17	18	11
	Minimum	3640	75	12	0		6. 1	45	4	13	16	8
	Maximum	12530	85	24	4		6. 7	55	10	20	21	14

cell leukemia. This seems likely since no granulocytosis was seen in the spontaneous leukemia that gave rise to this line of transplantation; and furthermore, growths of other transplantable neoplasms, *e.g.*, mammary carcinomas and subcutaneous sarcomas, have been found associated with a myeloid reaction.

Macroscopic Lesions

Figure 1 illustrates the pathological changes found at autopsy of the leukemic mice. The outstanding lesions have been enlargement of the liver, spleen, and lymph nodes, and infiltration of the perirenal tissue. The enlarged livers were of crisp consistence with the pattern of the lobules visible on the surface (*see* fig. 1); the enlarged spleens were dark red, mottled with smaller or larger white confluent nodules. In the later passages the mesenteric lymph node was immensely enlarged (fig. 1), and usually the thymus was more or less enlarged. Tumor growth at the site of inoculation (subcutaneously in the left flank) was observed only in rare cases. In some mice in which the leukemia took a prolonged course, one or more, but never all, lymph nodes contained larger or smaller cysts filled with serous or serosanguineous fluid (fig. 11).

The frequency of the macroscopic lesions is listed in table 1: In 40 leukemic mice enlargement of the thymus occurred in 85 percent; of the

liver, in 62 percent; of the spleen and perirenal tissue, in 92 percent; and of the lymph nodes, in 97 percent.

Microscopic Changes

The cells characteristic of the present line of leukemia are unquestionable plasma cells (figs. 7 to 10); their degree of maturity will be dealt with later in this paper.

Typical leukemic infiltrations were found in all sites of predilection in leukemia. In the lung, liver (fig. 12), and kidney, sharply demarcated, perivascular infiltrations were seen; in the thymus, spleen (fig. 13), perirenal tissue (fig. 14), and lymph nodes, massive infiltrations with complete obliteration of the normal structure of the organs were found.

The cystic changes in the lymph nodes (fig. 11) apparently were due to lymph congestion, since the cysts were lined with sinus endothelium, often more or less proliferating.

As is evident from table 1, infiltration of the liver, spleen, and lymph nodes was found in all mice, and in 24 leukemic mice examined microscopically, infiltration of the lungs was found in 88 percent, and infiltration of the kidneys in 66 percent.

Bone marrow of vertebrae was examined in 2 mice of the eighth passage in which the leukemia developed rather slowly. No leukemic infiltration of the bone marrow was observed in these 2 mice, in contradistinction to the finding in the bone marrow of the spontaneous plasma-cell leukemia of the present line, in which a nodule of plasma cells was found (fig. 3). In addition in 2 other leukemic mice no bone destruction was detected on the skeletal roentgenograms.

No systemic search for bone marrow changes has been carried out because such investigations were made in a series of leukemic mice of another paraprotein-developing line of plasma-cell leukemia without finding myeloma-like lesions. It may also be mentioned that roentgenograms of mice from several of our other lines of plasma-cell leukemia failed to show any bone destruction.

Differentiation Grade of the Plasma Cells

As stated, the spontaneous leukemia, from which the present transplantation line arose, was estimated to be a plasma-cell leukemia of differentiation grade II-III (fig. 2), according to the criteria briefly mentioned, and described and illustrated in our previous publications (*1, 8*).

In the first two transfer generations this degree of maturity (grade II-III) was maintained. In the third passage the plasma cells appeared very slightly more undifferentiated, estimated as grade III.

In the fourth, fifth, sixth, and seventh transfers the cytology of the plasma cells remained practically unchanged (grade III) (fig. 15), yet with minute, individual variations from mouse to mouse and from organ to organ in the same mouse.

In the eighth passage the leukemic cells undoubtedly became slightly more undifferentiated, more reticulosarcoma-like, and richer in mitoses (estimated grade III-IV, fig. 16).

This dedifferentiation was not evident in imprint preparations of infiltrated organs nor in smears of blood with plasma cells.

Sections of the organs from a mouse of the second, third, fourth, seventh, and eighth transfer generations were examined in specially stained preparations.[6] When stained by the Unna Pappenheim method, the leukemic plasma cells were highly pyroninophilic, particularly the more mature forms. In the plasma cells of differentiation grade III-IV, the pyroninophilic stain was less intense, but the sections gave a general impression of marked pyroninophilia because these cells contained more ample amounts of cytoplasm. The PAS-staining was negative in plasma cells of all differentiation grades. No signs of amyloidosis were observed. In reticulin-stained preparations a scarce, very fine network was seen between the plasma cells of all differentiation grades, but without any relation to the neoplastic cells, which showed no evidence of reticulin production. No doubt the reticular network has to be considered as belonging to the normal connective tissue left between the tumor cells.

Correlation of the Development of Hematological, Serological, and Histological Changes

Forty-five mice were inoculated subcutaneously with leukemic tissue from a mouse of the fourth passage. At 7-day intervals 2 of these mice were killed, and hematological, serological, and histological examinations were made. Since the leukemia developed with varying rapidity, the lifetime of the killed mice could in no way be indicative of the dimensions of the leukemic lesions; consequently, the macroscopic and microscopic lesions had to be used as a basis for the estimation of the extent of the leukemic process.

In table 3, nine mice, arranged according to increasing histological changes, are listed, together with the corresponding hematological and serological findings. It will be seen that simultaneously with the earliest manifestations of the leukemic lesions in the organs, the granulocytosis began and became more and more pronounced as the disease intensified. Plasma cells were not seen in the blood until the leukemic infiltrations had become extremely heavy and widespread.

It also may be noticed in table 3 that the paraprotein was released into the blood when the leukemic lesions had developed only to a moderate degree, but it increased in amount with further development of the leukemic process. This accords well with the fact that paraprotein in serum from leukemic mice has usually been observed for a considerable length of time before the mice died.

Examination of urine.—Paper electrophoretic investigations have been carried out on urine from several leukemic mice of the present line of

[6] These investigations were undertaken by H. E. Christensen M.D., The Laboratory for Rheumatic Research, University Institute of Pathological Anatomy, Copenhagen.

TABLE 3.—*Correlation of hematological, serological, and histological findings during development of plasma-cell leukemia, transplantation line 20*

Mouse No.	Survival time (*days*)	Examination of lesions	Leukemic infiltrations in:							Blood picture:				Electrophoretic determination:				
			Thymus	Lung	Liver	Spleen	Kidney	Perirenal tissue	Lymph nodes	Lymphocytes	Granulocytes	Monocytes	Plasma cells	Albumin	α_1-Globulin	α_2-Globulin	β-Globulin	γ-Globulin
7780	33	Gross	−	−	−	−	−	−	−	80	15	5		55	7	11	17	10
		Microscopic		−	−	−	−		−									
7815	54	Gross	−	−	−	−	−	−	−	46	48	6		54	8	16	17	5
		Microscopic		−	+	++	−		+									
7803	116	Gross	−	−	−	−	−	−	++	48	40	12		50	5	17	20	8
		Microscopic		−	+	++	−	+	++									
7797	61	Gross	−	−	−	+	−	−	++	42	47	11		36	9	17	21	17
		Microscopic		−	+	+	+	−	+++									
7798	61	Gross	−	−	−	++	−	++	++	26	60	14		35	12	18	30	5
		Microscopic		−	+	++	−		++									
7814	54	Gross	++	−	+	++	−	++	++	37	55	8		34	13	19	31	3
		Microscopic		−	+	+++	−		+++									
7789	47	Gross	−	−	++	+++	−	+++	+++	33	57	10		31	8	24	31	6
		Microscopic		++	++	+++	−	+++	+++									
7802	147	Gross	+	−	+++	+++	−	++	+++	12	84	2	2	33	6	17	33	11
		Microscopic		−	+++	+++	−	++	+++									
7811	80	Gross	++	−	++	+++	−	+++	+++	9	19	7	65	24	7	16	36	17
		Microscopic		++	+++	+++	+	+++	+++									
Ten normal mice		Mean								78	20	2		48	6	17	18	11
		Minimum								75	12	0		45	4	13	16	8
		Maximum								85	24	4		55	10	20	21	14

leukemia. No paraprotein could be detected but varying amounts of albumin, which is known to be excreted physiologically in mice, particularly in males, were observed.

Discussion

So far only two transplantable plasma-cell neoplasias associated with the development of abnormal serum-protein fractions have been described: the myeloma-like disease in C3H mice reported by Potter *et al.* (*9*), and the present leukemic growth in (CBA × DBA/2)F_1 mice. Except for the production of paraprotein these neoplasias differ in most respects. The line described by Potter *et al.* is a locally growing, late metastasizing plasmocytoma, with the development of bone destruction. The line described in this paper shows typical leukemic characteristics, outflow of plasma cells in the blood, widely disseminated leukemic infiltrations, and no tumor at the site of inoculation. A similar plasmocytoma, only without the occurrence of paraprotein, and several other C3H plasmocytomas not transplanted or unsuccessfully transplanted have also been described (*3–7*). In this laboratory, numerous transplantation lines of plasma-cell leukemias in (CBA × DBA/2)F_1 mice with and without the production of paraprotein have been established (*14*), but in our mice no localized plasmocytoma has occurred. Thus, the type of plasma-cell neoplasia that will develop seems a matter of genetic constitution.

Both types occur in human beings—multiple myeloma being observed far more frequently than plasma-cell leukemia. Bichel *et al.* (*29*) calculated the ratio of incidence between plasma-cell leukemia and multiple myeloma to be 1:50, and the ratio between plasma-cell leukemia and other forms of leukemia to be 1:250. These data were collected from the notifications to the Danish Cancer Registry, including the whole Danish population of 4.1 million inhabitants, from 1943 through 1947.

The paraprotein of the present line of plasma-cell leukemia was found in paper electrophoresis to belong to the β-globulin fraction, the electrophoretic pattern being quite similar to that of human β-myeloma. In immunoelectrophoretic studies the paraprotein was revealed to be a γ-protein of β-mobility. This is also in accordance with the findings in human myeloma (*30*), in which not only γ- but also some M- and β-myelomas have been observed, immunoelectrophoretically, to derive their paraprotein from corresponding electrophoretic regions of the γ-bow.

Not all human myelomas and plasma-cell leukemias show an increased amount of total serum protein; thus there is no discrepancy in this point between the human disease and the present plasma-cell leukemia, in which normal total serum protein was found.

It is evident that the amount of paraprotein in the serum of leukemic mice decreased in the later transfer generations, as did the survival time; at the same time the histological picture of the plasma cells changed to a slightly more undifferentiated form, from grade II-III to grade III-IV. Whether this slight dedifferentiation of the leukemia through the passages

is responsible for the decrease of the paraprotein fraction in serum is undecided, but it may be so, even if there was no absolute correlation between the juncture of the alterations of the serum patterns and of the cytology. We have observed the fading of the paraprotein fraction in later passages of more transplantation lines of plasma-cell leukemia; thus, it seems as if this fading is a rather regularly occurring feature in the development of our transplantation lines of plasma-cell leukemia. Further discussion of this problem is therefore postponed until more of these lines have been thoroughly investigated.

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PLATE 50

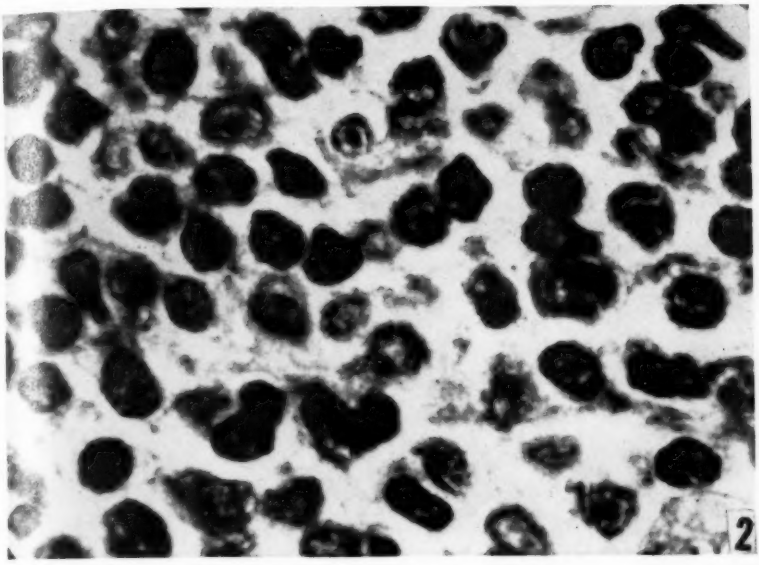
FIGURE 1.—Mouse with plasma-cell leukemia, transplantation line 20.

493212—59——9

PLATE 51

FIGURE 2.—Plasma cells in renal, perivascular infiltration from mouse No. 5679—the spontaneous case of plasma-cell leukemia giving rise to transplantation li[illegible]20. Hematoxylin and eosin. × 1,300

FIGURE 3.—Bone marrow with nodule of plasma cells from mouse No. 5679—the spontaneous case of plasma-cell leukemia. Hematoxylin and eosin. × 150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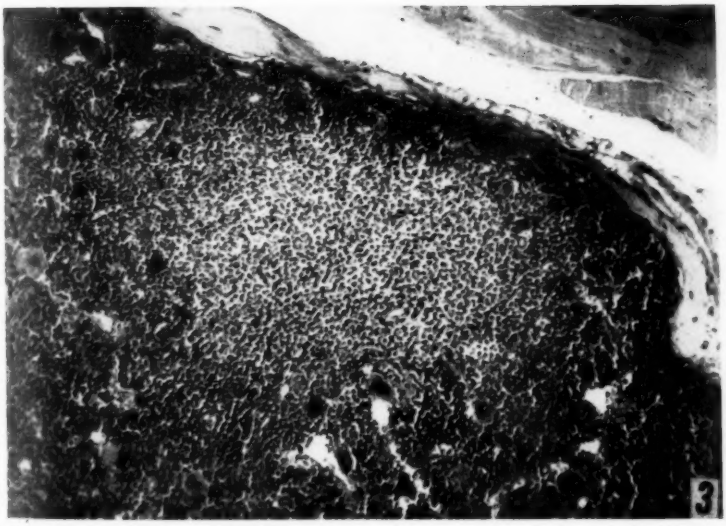
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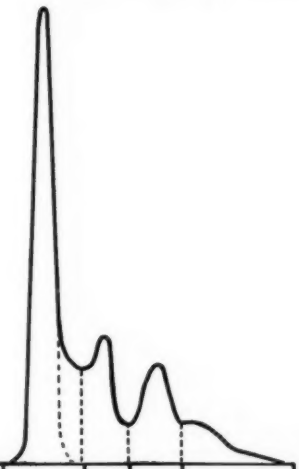
PLATE 52

FIGURE 4.—Paper electrophoretic patterns and scanning diagrams.

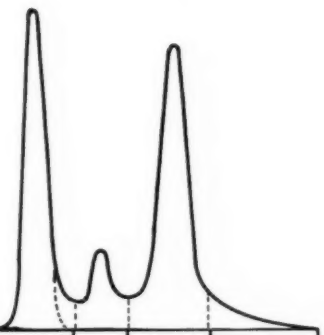
a) Normal mouse serum.

b) Serum from mouse No. 7411 of the fourth passage of plasma-cell leukemia, transplantation line 20.



Total prot.	alb. + α_1		α_2	β	γ
rel. %	48	6	17	18	11
6.5 gms %	3,1	0,4	1,1	1,2	0,7

a



Total prot.	alb. + α_1		α_2	β	γ
rel %	36	3	12	43	6
6.4 gms %	2,3	0,2	0,8	2,7	0,4

b 4

PLATE 53

FIGURE 5.—Immunoelectrophoretic patterns. × 2.5

a) Conventional agar-gel electrophoresis of serum from mouse No. 7411 of the fourth passage of plasma-cell leukemia, transplantation line 20.

b) Precipitation pattern of normal mouse serum.

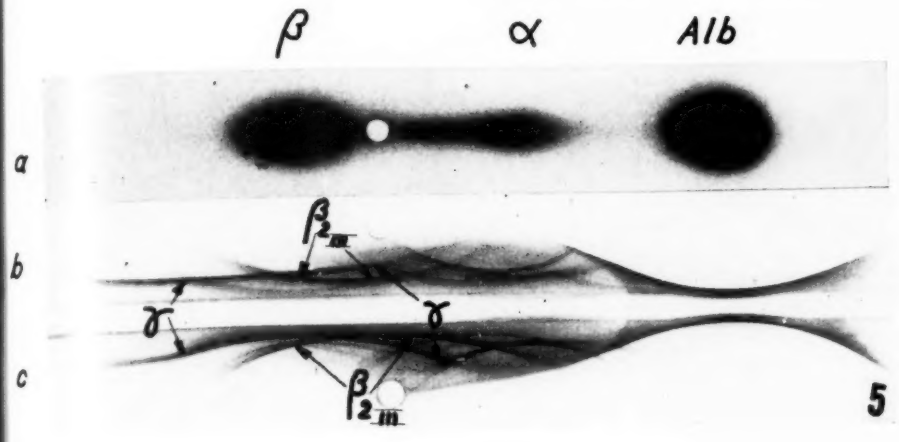
c) Precipitation pattern of serum from mouse No. 7411.

FIGURE 6.—Starch-gel electrophoretic patterns. × 3

a) Normal mouse serum.

b) Serum from mouse No. 2182 of the sixth passage of plasma-cell leukemia, transplantation line 20.

c) Serum from mouse No. 7411 of the fourth passage of plasma-cell leukemia, transplantation line 20.


β
α
Alb
a
b
c
$\beta_{2_{III}}$
γ
γ
$\beta_{2_{III}}$
5

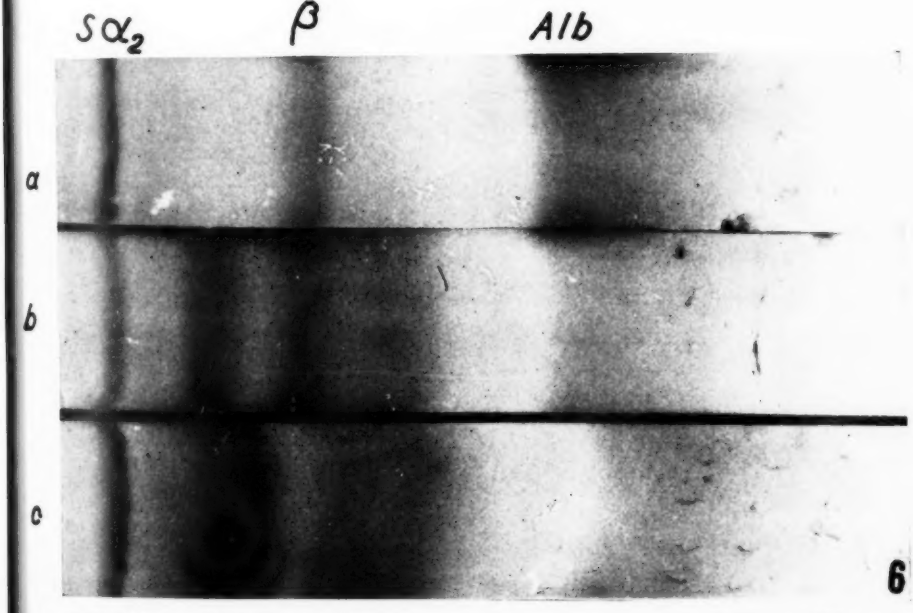
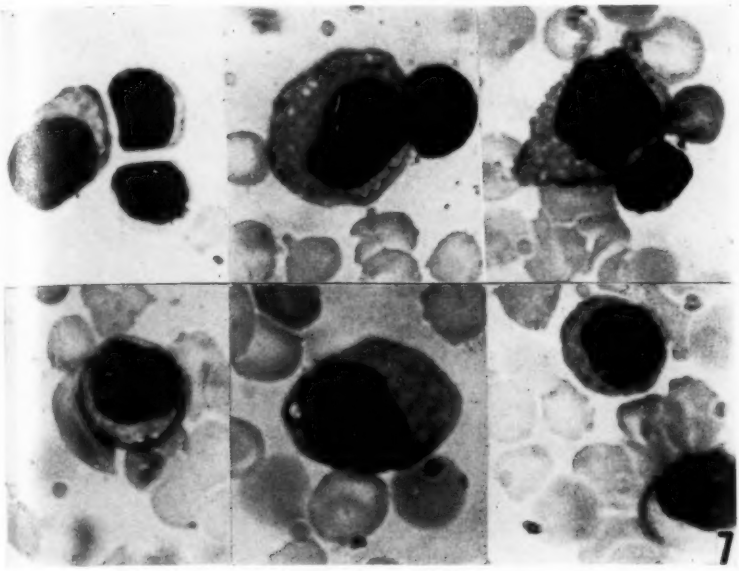

$S\alpha_2$
β
Alb
a
b
c
6

PLATE 54

FIGURE 7.—Plasma cells in Giemsa-stained blood smear from mouse with plasma-cell leukemia. × 1,400

FIGURE 8.—Highly polymorphous plasma cells in Giemsa-stained imprint preparation of lymph node from mouse with plasma-cell leukemia, transplantation line 20. × 380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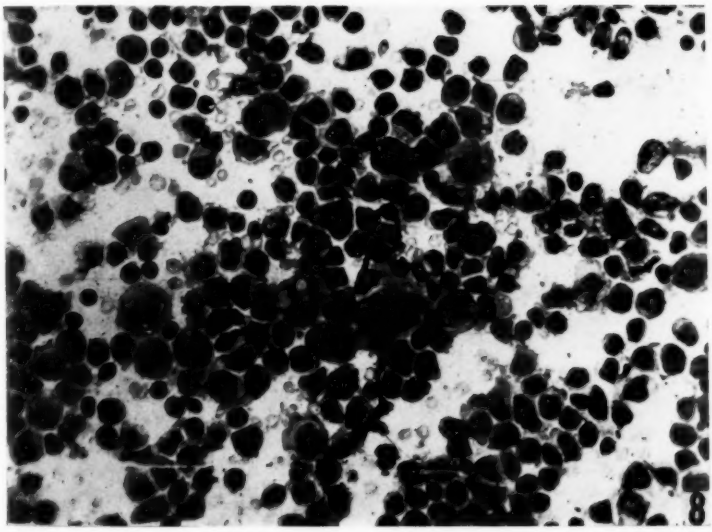
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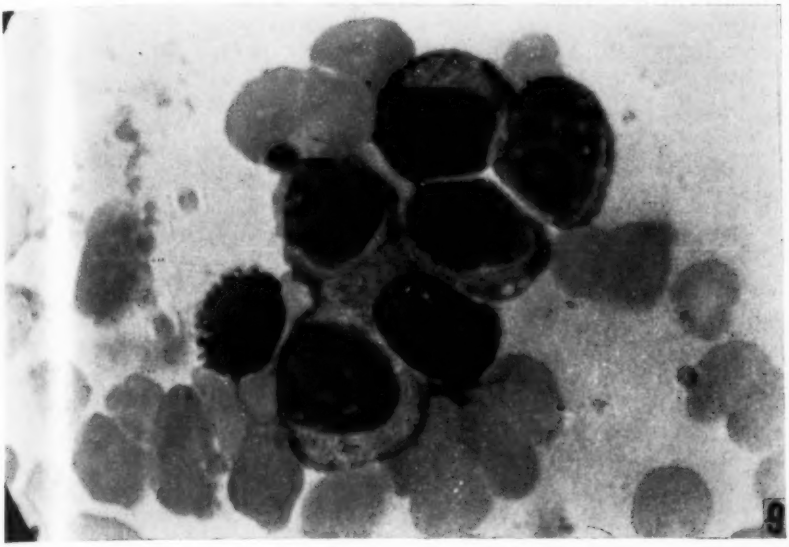
PLATE 55

FIGURE 9.—Plasma cells in Giemsa-stained blood smear from mouse with plasma-cell leukemia. × 1,800

FIGURE 10.—Plasma cells in Giemsa-stained imprint preparation of spleen from mouse with plasma-cell leukemia. × 1,800



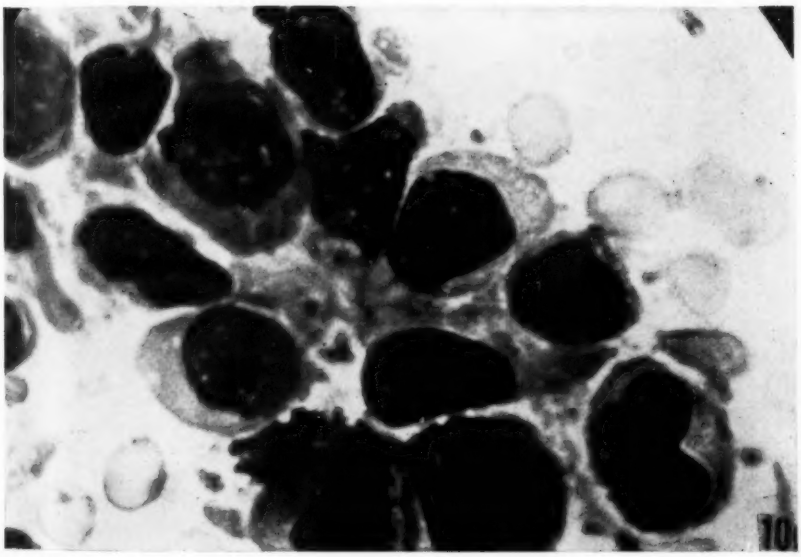


PLATE 56

FIGURE 11.—Axillary lymph node with cystic changes from mouse with plasma-cell leukemia. Hematoxylin and eosin. × 10

FIGURE 12.—Infiltration of liver from mouse with plasma-cell leukemia. Hematoxylin and eosin. × 70

FIGURE 13.—Spleen from mouse with plasma-cell leukemia. Differentiation grade III. Hematoxylin and eosin. × 285

FIGURE 14.—Perirenal infiltration from mouse with plasma-cell leukemia. Hematoxylin and eosin. × 70

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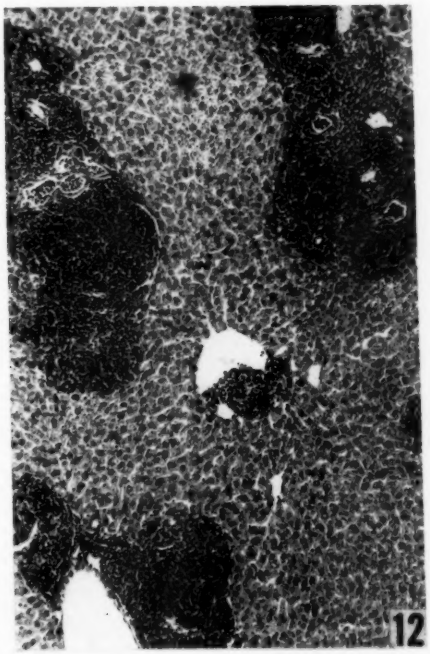
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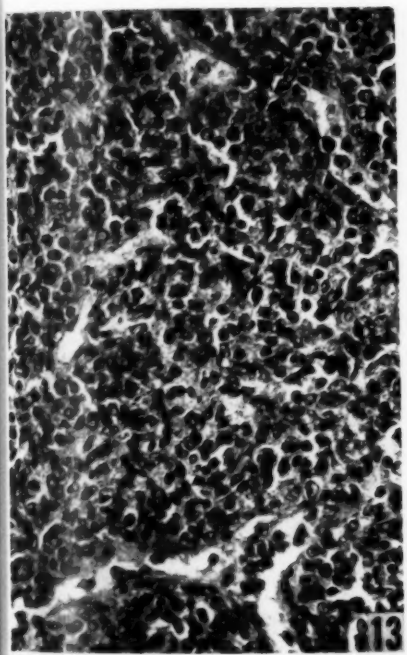
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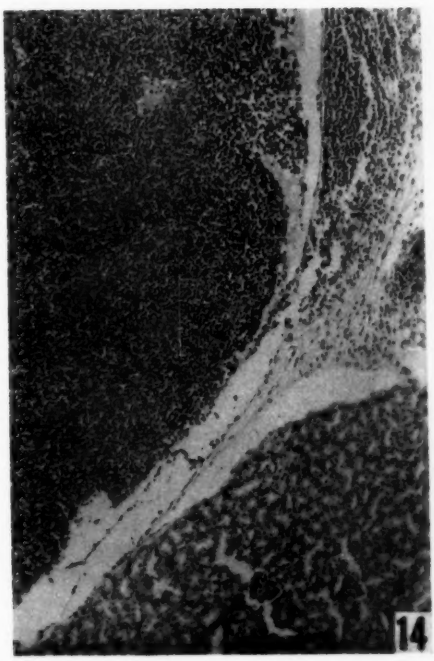
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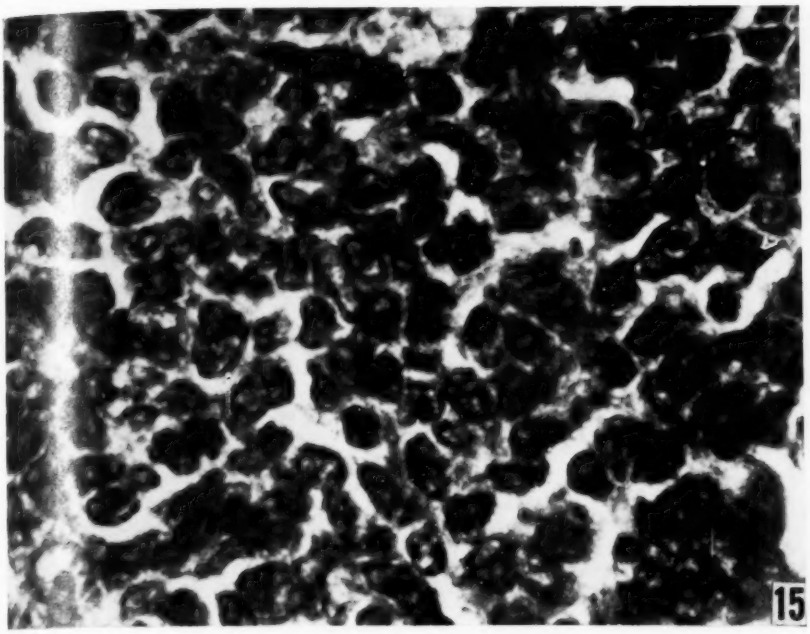
PLATE 57

FIGURE 15.—Plasma cells in perivascular infiltration of the liver from mouse with plasma-cell leukemia. Differentiation grade III. Hematoxylin and eosin. × 1,300

FIGURE 16.—Plasma cells in perivascular infiltration of the liver from mouse with plasma-cell leukemia. Differentiation grade III-IV. Hematoxylin and eosin. × 1,300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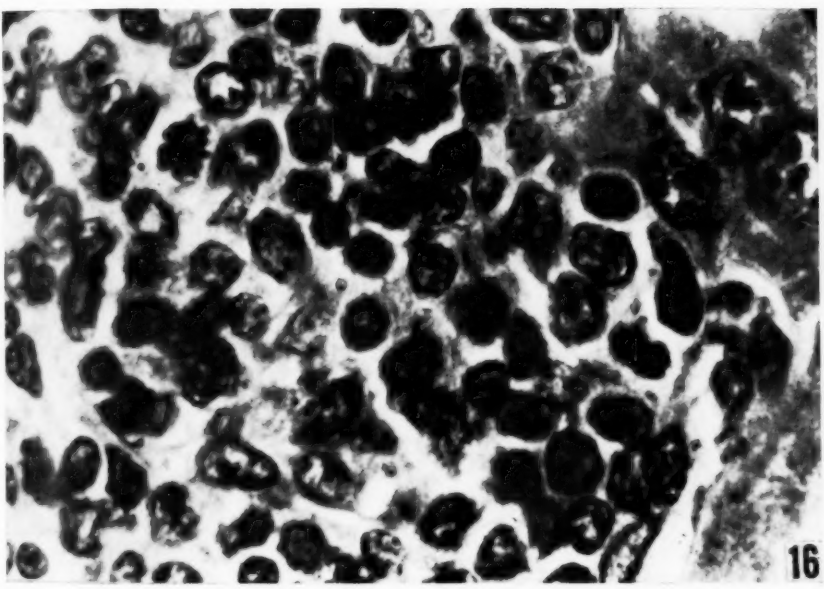
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A Preliminary Report on the Effect of Thyroid Analogues in Neoplastic Diseases in Man [1,2]

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Summary

Fourteen patients with cancer were treated with thyroid-hormone derivatives: 5 received triiodothyronine, 5, tetraiodothyropropionic acid, 2, triiodothyropropionic acid, 1 received all three derivatives, and 1, the latter two agents. There was no objective improvement in any patient except possibly in 1 patient with acute granulocytic leukemia who had a transient clinical and hematologic improvement associated with triiodothyronine treatment, and 1 patient with teratochoriocarcinoma of the testis in whom a fall in urinary gonadotrophins accompanied treatment with triiodothyropropionic acid. No evidence of tumor regression or cellular differentiation resulting from treatment was observed. Tetraiodothyropropionic acid was found to have effects qualitatively similar to thyroxine in humans. It raised the basal metabolic rate, depressed the radioactive iodine uptake, and increased the protein-bound iodine.—**J. Nat. Cancer Inst. 22: 543–550, 1959.**

Although much is known about the influence of estrogens, androgens, and adrenocortical hormones on human cancers, very little information is available concerning the effects of thyroid hormones. Experimental studies of their effect on animal tumors have been conflicting. Some investigators reported that thyroid hormones caused inhibition of tumor growth (*1–4*) but others were unable to demonstrate an effect upon tumor growth (*5–7*). The treatment of human thyroid cancer with thyroid hormones is well established (*8, 9*). Thyroid hormones inhibit production of thyrotrophic hormone, which is thought to accelerate growth of thyroid cancer. However, few reports are available on the treatment of human nonthyroid cancers with thyroid hormones. Loeser reported a beneficial effect of massive doses of desiccated thyroid in 5 women with inoperable breast and ovarian cancer (*10*). Supplementary thyroid-hormone

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[4] We wish to thank Glenn E. Sheline, M.D., Department of Radiology, for the radioactive iodine uptake studies, and Barbara Hughes, Marie Doherty, and Rose Lee, for their technical assistance during these studies.

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therapy was advocated in the management of patients with metastatic breast cancer (*11*), and as a supplement to estrogens in the treatment of carcinoma of the prostate (*12*).

The evidence suggesting a relationship between thyroid dysfunction and neoplasia (*7, 10, 13–18*) and the reported benefits of thyroid hormones in human carcinoma suggested to us that further trials should be undertaken with thyroid therapy on human malignancies. It has been hypothesized that thyroid hormones might exert an influence on neoplasms either by producing some alteration in metabolism of the tumor or by promoting maturation of the undifferentiated cancer cells (*8, 18, 19*). The recent developments in the field of thyroid-hormone chemistry have provided several thyroid analogues which are reported to have marked dissociation between metabolic activity and maturative activity as evidenced by their ability to promote metamorphosis (*20–23*). The thyroid analogues selected for this study were triiodothyronine, which has very great metabolic activity, and the thyropropionic acid derivatives, which are very active in promoting amphibian metamorphosis but have been reported to possess very little metabolic activity (*20–22*, table 1). In addition to evaluation of their possible antineoplastic effects, certain metabolic effects produced by these compounds were also studied.

TABLE 1.—*Comparative biological activity for analogues of thyroxine**

Compound	Relative biologic activity	
	Metabolic rate	Metamorphosis
L-Thyroxine	1.0	1
Triiodo-L-thyronine	3–5.0	5–20
Tetraiodothyropropionic acid	0.1	21–80
Triiodothyropropionic acid	0.5	300

*Reference *20–22*.

Materials and Methods

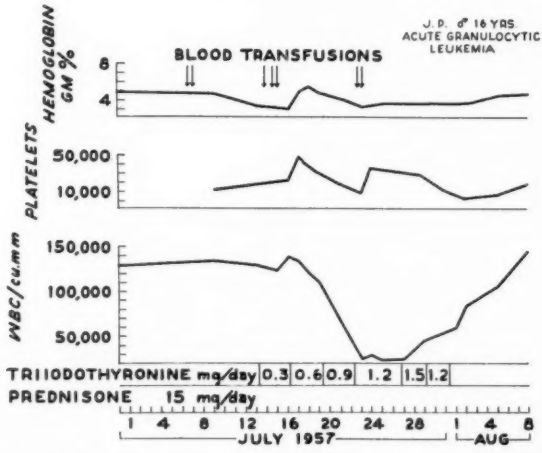
Thyroid analogues were employed in the treatment of patients with advanced cancer. The sex, age, diagnosis, duration and extent of disease, prior therapy, and general condition of the patient at the time therapy with thyroid analogues was begun are shown in table 2. Five patients received triiodothyronine, 5, tetraiodothyropropionic acid, and 2, triiodothyropropionic acid. The latter 2 and 1 other patient received all three thyroid analogues sequentially. The thyropropionic acids [5] were dissolved in a slightly alkaline (*p*H 9 to 10) 0.9 percent NaCl solution and administered intramuscularly. The daily dose of tetraiodothyropropionic acid varied between 1.2 and 30 mg.—most patients received 10 mg. daily. Daily dose of triiodothyropropionic acid was 5 mg. The daily oral dose of 3,5,3′-L-triiodothyronine (Cytomel®) was from 0.2 to 1.5 mg.—most patients received 0.6 mg. daily. Antibiotics, transfusions, and other addi-

[5] The tetraiodothyropropionic acid and triiodothyropropionic acid used in this study were obtained from Cyclo Chemical Corporation, Los Angeles, Calif.

tive therapy were employed when clinically indicated. Certain tests relating to thyroid function, such as blood cholesterol, PBI, BMR, and radioactive iodine uptake were obtained at varying intervals during treatment with thyroid analogues. Most patients were observed in the hospital for at least 1 week before treatment was started. Hematologic studies of most patients were made at least three times weekly. Chest X rays of patients with pulmonary metastases were obtained at weekly intervals. Quantitative urinary gonadotrophin measurements were made in 1 patient with teratochoriocarcinoma of the testes.

Results

Six patients received triiodothyronine: 2 with acute monocytic leukemia, and 1 each with acute granulocytic leukemia, acute lymphatic leukemia, chronic granulocytic leukemia, and multiple myeloma (table 2). The addition of large doses of this drug to the therapeutic regimen produced no effect upon the course of the disease, except in 1 patient. This was a 16-year-old male with acute granulocytic leukemia, who showed marked subjective clinical improvement associated with a fall in the total white blood count for a period of 12 days. No effect was seen on hemoglobin or platelets. His response to therapy is summarized in text-figure 1. All patients receiving triiodothyronine developed varying degrees of thyrotoxicity as indicated by tachycardia, nervousness, diaphoresis, and



TEXT-FIGURE 1.—Acute granulocytic leukemia in a 16-year-old male. Diagnosis was made on May 27, 1957, at which time symptoms of fatigability, bruisability, and pallor had been present for 2 months. Treatment consisted of blood transfusions and prednisone, 15 mg. per day. The course of the disease, from July 1 to August 8, is shown above. The patient experienced subjective improvement associated with a fall in the white blood count during the 12-day period from July 16 to 28, but then became worse and expired on August 21, 1957. The hemoglobin and platelet levels failed to improve and no alteration in the character of the differential count was noted.

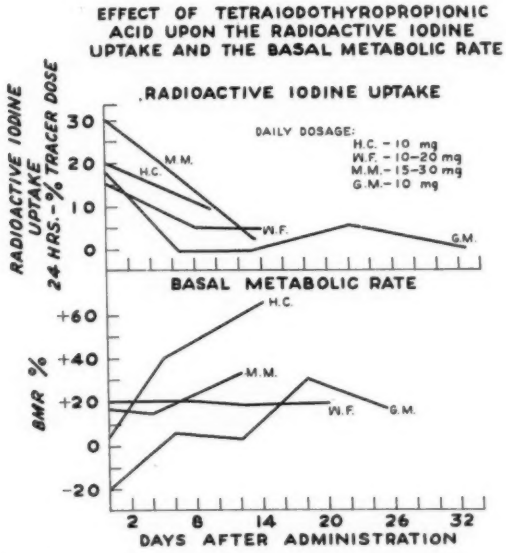
TABLE 2.—*Patients and treatment schedule*

Patient	Sex	Age	Diagnosis	Duration	Previous treatment	Condition	Treatment	Dose per day (*mg.*)	Days used	Additive treatment
M.E.	Male	7	Acute lymphatic leukemia	13 months	6-MP, amethopterin, prednisone	Poor	Triiodothyronine	0.2–0.6	20	Blood, prednisone
W.F.	Male	72	Chronic granulocytic leukemia	8 years	Myleran	Good	Triiodothyronine, tetraiodothyropropionic acid, triiodothyropropionic acid	0.3 10–20 5	11 22 18	
R.K.	Female	63	Acute monocytic leukemia	1 month	Blood, prednisone, 6-MP	Critical	Triiodothyronine	0.6	4	Blood, prednisone
W.L.	Male	56	Multiple myeloma	6 months	None	Fair	Triiodothyronine	0.6	10	
J.P.	Male	16	Acute granulocytic leukemia	2 months	Blood, prednisone	Poor	Triiodothyronine	0.3–1.5	19	Blood, prednisone
R.S.	Female	45	Acute monocytic leukemia	9 months	Blood, prednisone	Poor	Triiodothyronine	0.3–0.9	9	Blood, prednisone
H.C.	Male	55	Adenocarcinoma of rectosigmoid with liver and lung metastases	2 years and 7 months	Surgery	Poor	Tetraiodothyropropionic acid	10	16	
I.E.	Male	70	Lymphosarcoma	10 years	Nitrogen mustard, P^{32}, X ray	Poor	Tetraiodothyropropionic acid	1.2–15	7	
M.K.	Female	29	Carcinoma of breast with brain and lung metastases	4 years	Radical mastectomy, adrenalectomy, oophorectomy	Poor	Tetraiodothyropropionic acid	10–20	24	

G.M.	Female	75	Papillary adenocarcinoma of thyroid with lung metastases	13 years	Surgery	Fair	Tetraiodothyropropionic acid	10	43	
M.M.	Female	61	Carcinoma of lung	15 months	None	Poor	Tetraiodothyropropionic acid	15–30	9	
M.C.	Male	2	Neuroblastoma with lung metastases	16 months	Surgery, vitamin B_{12}	Poor	Triiodothyropropionic acid	5	13	
B.Y.	Female	52	Acute monocytic leukemia	13 months	Prednisone, 6-MP	Poor	Triiodothyropropionic acid	5	12	Prednisone, blood
J.B.	Male	34	Teratochoriocarcinoma of testis with lymph-node and lung metastases	7 months	Surgery, amethopterin	Fair	Triiodothyropropionic acid, tetraiodothyropropriomic acid	10	16 50	

elevation of the basal metabolic rate. No patient showed evidence of hematopoietic toxicity.

Nine patients received thyroproprionic acids but only 1 demonstrated objective or subjective evidence of clinical improvement. Five patients received tetraiodothyropropionic acid, 2 received triiodothyroproprionic acid, and 2 received both drugs. The patients receiving thyroproprionic acids developed only mild degrees of thyrotoxicity as compared with those who received triiodothyronine. Tetraiodothyroproprionic acid was observed to produce effects qualitatively similar to thyroxine, *e.g.*, elevation of the basal metabolic rate (text-fig. 2), elevation of the protein-bound iodine (table 3), and lowering of the radioactive iodine uptake (text-fig. 2).



TEXT-FIGURE 2.—Effect of tetraiodothyroproprionic acid upon the radioactive iodine uptake and the basal metabolic rate.

TABLE 3.—*Changes in blood cholesterol and serum protein-bound iodine induced by tetraiodothyroproprionic acid*

Patient	Cholesterol (*mg./100/ml.*)		PBI (*μg./100/ml.*)	
	Before	After	Before	After
W.F.	227	328	6. 2	111. 5*
M.K.	205	217	5. 6	>40
H.C.	233	211	6. 6	>40
G.M.	330	239	5. 3	>40
M.M.	238	229	7. 7	>40
J.B.	288	227	9. 1	>40

*This specimen was diluted before being tested. All other specimens were not diluted, therefore true values were not determined, but all were over 40 μg. per 100 ml.

It lowered slightly the blood cholesterol in 4 of the 6 patients (table 3). No significant effects were produced on the hematopoietic system.

In 1 patient (J.B.) with teratochoriocarcinoma of the testis, a significant reduction in the quantitative urinary chorionic gonadotrophin resulted from treatment with 10 mg. per day of triiodothyroproprionic acid. In this patient the gonadotrophin titer fell from the control value of 40,000 mouse units per liter per 24 hours to 1,000 mouse units per liter after 2 weeks of therapy. When the drug was discontinued, the titer rose to 10,000 mouse units per liter within several days. At this point the patient was placed on tetraiodothyroproprionic acid, 20 mg. per day, for a period of 50 days. During this period, the gonadotrophin titer was maintained at 10,000 mouse units per liter and rose to 30,000 mouse units per liter a week after the drug was withdrawn. This reduction in urinary gonadotrophins was not associated with other evidence of clinical improvement.

Discussion

Except for 2 patients, no objective effects upon the neoplastic process were produced by the thyroid-hormone analogues during the period of administration. The temporary clinical and hematologic improvement, possibly a spontaneous remission, elicited in the patient with acute granulocytic leukemia is difficult to evaluate. No effects were observed to suggest that maturation of the undifferentiated leukemic or tumor cells had occurred as a result of treatment with large doses of these compounds, although longer periods of therapy may be warranted. It is known that it may take several months to correct the anemia of myxedema after instituting thyroid therapy (*23*).

The effects of tetraiodothyroproprionic acid upon the basal metabolic rate, protein-bound iodine, blood cholesterol, and radioiodine uptake were found to be qualitatively similar to those produced by thyroxine. This is what might be expected from the known effects of these thyroid analogues in animals (*20–22*). The fall in urinary gonadotrophin titer in the patient with teratochoriocarcinoma may indicate inhibition of the choriocarcinoma component of this mixed tumor. The markedly elevated protein-bound iodine levels encountered probably reflect the binding of this compound to the serum proteins similar to that occurring with thyroxine.

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The Deoxyribonucleic Acid (DNA) Content of Sternberg-Reed Cells of Hodgkin's Disease [1, 2]

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Summary

The DNA content of Sternberg-Reed cells of Hodgkin's disease was determined by Feulgen cytophotometry. An increased DNA content per cell nucleus above the diploid ($2n$) level was found which corresponded to the nuclear size and degree of nuclear lobulation. The findings were considered as evidence in support of the concept that the Sternberg-Reed nuclei undergo endomitosis and polyploidization during their development from the reticulum cells.—**J. Nat. Cancer Inst. 22: 551–554, 1959.**

The Sternberg-Reed cell is considered by pathologists to be the pathognomonic feature of Hodgkin's disease (*1*, *2*). Many studies have been reported on the morphology of the Sternberg-Reed cells in tissue sections and lymph-node imprints (*3–7*). Of particular interest are the abnormalities of nuclear morphology, such as giant nuclei, multiple nuclei, inequality of nuclear size, and large nucleoli. The few histochemical studies on these cells reported employing the Feulgen stain have suggested that at certain morphologic stages the nuclei possess an increased content of deoxyribonucleic acid (DNA) (*8*, *9*). These reports suggested that a semiquantitative study of the DNA content of the Sternberg-Reed cell nuclei might be of value in interpreting the mechanism of their method of formation, since the DNA of the cell nucleus is intimately allied to chromosome structure and mitotic activity (*10*). Nuclear DNA measurements have been employed for the detection of quantitative alterations in chromosome numbers and in disturbances of DNA chromosome relationships in a variety of malignant cells (*11–13*). In the present study, the DNA content of individual Sternberg-Reed cells from lymph-node imprints was determined by the microspectrophotometric technique, with the Feulgen stain for DNA. For purposes of comparison, additional

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[2] This investigation was supported in part by research grant CY-3422(C1)CY from the Division of Research Grants, National Institutes of Health, Public Health Service, and by the Dorothy H. and Lewis Rosenstiel Foundation.

studies were made of the DNA in the nuclei of reticulum-cell sarcoma cells and normal megakaryocytes from the bone marrow.

Materials and Methods

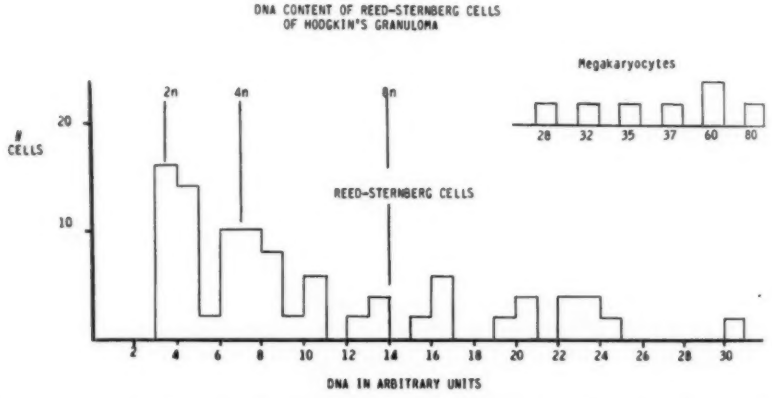
Sternberg-Reed cells were found in imprints from lymph nodes of 5 patients diagnosed histologically as Hodgkin's granuloma. The imprints were fixed in absolute methyl alcohol and stained with the Feulgen stain; the microspectrophotometric apparatus and technique employed were similar to those described in previous studies (*11*, *12*). The amount of DNA Feulgen complex in the nucleus was obtained in arbitrary units from the formula $DNA = E \times$ nuclear area/0.6, where E is the optical density of the stained nucleus.

The nuclei used for measurement were selected for their homogeneous staining in order to minimize the error introduced by inhomogeneous chromatin distribution.

Results

The DNA was determined in individual normal lymphocytes, and the average of the distributed values was designated as the diploid $2n$ content. This value, shown as a vertical line in text-figure 1, was used as the reference standard to establish the tetraploid and octoploid DNA levels.

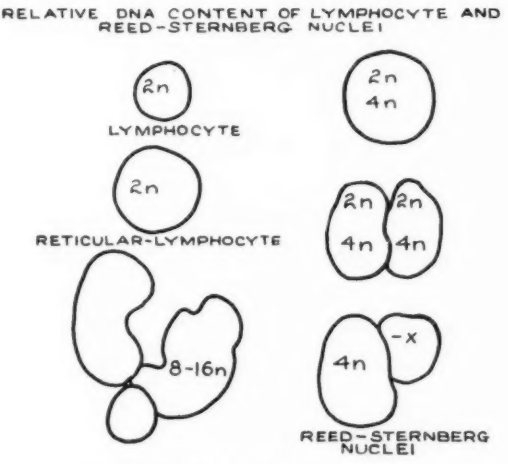
The distribution of DNA values in 44 individual Sternberg-Reed nuclei is shown in text-figure 1. The values ranged from the $2n$ DNA level to very high values. For purposes of the present study, cells whose nuclear diameter averaged over 14μ were designated as reticular lymphocytes or reticulum cells (*14*). In the majority of these cells, the DNA values were found to be diploid, but, in some, the amounts were twice the diploid value. With increasing nuclear diameter and lobulation, the nuclear DNA content per cell was found to increase. In highly multinucleated cells, the total DNA per cell was markedly increased, and unequal amounts



TEXT-FIGURE 1.—The distribution of individual DNA values (Feulgen units) in the nuclei of Sternberg-Reed cells of Hodgkin's disease.

of DNA were distributed between the nuclei in many binucleated and multinucleated cells. The variations found in nuclear DNA distribution are shown in text-figure 2.

In the reticulum-cell sarcoma nuclei measured, the DNA content was found to fall in the diploid range, except in multinucleated forms where the total DNA per cell was increased in proportion to the number of nuclear lobes and fell into the same range of values as obtained in the Sternberg-Reed cells. In the few mature megakaryocytes measured, the DNA



TEXT-FIGURE 2.—The relative DNA content of various forms of Sternberg-Reed nuclei as compared with the DNA content of normal diploid lymphocytes.

content was markedly elevated (text-fig. 2). The DNA values overlapped with those obtained in the Sternberg-Reed cells.

Discussion

These findings demonstrate that the Sternberg-Reed cells of Hodgkin's granuloma may contain increased amounts of DNA as compared with normal diploid lymphocytes and reticular lymphocytes. These results confirm the observations of Ackerman, Knouff, and Hoster (*8*), and of Orlandi and Rottino (*9*), who reported an increased DNA quantity in some forms of Sternberg-Reed nuclei as suggested by visual comparisons with normal reticulum cells. It is believed that the giant forms develop from the reticulum cells of the lymph-node tissue and that the nuclei undergo progressive enlargement, lobulation, and, ultimately, pyknosis and disintegration (*4, 15*). The present findings indicate that these changes in nuclear morphology are associated with an increasing content of DNA. Whether this increase is a result of increased chromosome numbers or of endomitotic DNA synthesis and the formation of polytene chromosomes, as reported in other neoplastic cells, is not certain.

It is of interest that the DNA content of the reticulum-cell sarcoma nuclei was similar to that in the Sternberg-Reed nuclei. Megakaryocytes, which occasionally may be confused on morphologic grounds with Sternberg-Reed cells, were found to contain much greater amounts of DNA than do Sternberg-Reed cells. This criterion does not appear to be of significant practical value in differential diagnosis, as it would be anticipated that cells with more complex nuclear lobulation would contain amounts of DNA greater in quantity than that in Sternberg-Reed cells. Megakaryoblasts or promegakaryocytes in the less lobulated nuclei were not studied.

The present findings support the morphologic impression that Sternberg-Reed nuclei undergo endomitosis and polyploidization in their development from the reticulum cells.

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"Atypical" Cells in the Blood of Cancer Patients—Differentiation From Tumor Cells [1, 2]

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Summary

"Atypical" and immature cells are frequent in the blood of patients with cancer. This conclusion is based on the examination of the blood from 525 subjects with cancer and from 132 subjects without cancer. Many cells are difficult to differentiate from tumor cells. Representative cells are illustrated, and their distribution and some of their morphological features are discussed.—**J. Nat. Cancer Inst. 22: 555–565, 1959.**

When over 3,000 blood samples were examined for tumor cells, we observed a fairly large number of cells which we labeled "atypical" (*1*, *2*). Many of these cells are easily differentiated from tumor cells, but their presence, in significant numbers, in blood obtained from the veins that drain certain organs, in cancer patients, makes them of special interest. On the other hand, some of the "atypical" cells present a problem in differentiation from cancer cells. The majority of abnormal and immature hematopoietic elements usually does not present much of a problem in differentiation to the experienced person, especially those versed in bone-marrow morphology. Other cells can be identified, with some certainty, as endothelial, epithelial, or fibroblastic in origin. We have been impressed by observing cells whose identity remains obscure in the blood of cancer patients. The major purpose of the present communication is to draw the attention of those interested in the study of the cellular elements of the blood to the wide variety of "atypical" cells which may be in the blood.

Methods and Materials

The methods for collecting and processing blood, and the preparation of buffy-coat smears for the examination of tumor cells, as well as the differentiation between "positive" tumor cells present and "atypical" cells, have been described in previous communications (*1–3*). Comparison

[1] Received for publication September 18, 1958.

[2] This work has been supported in part by research grant C-3435 from the National Cancer Institute, National Institutes of Health, Public Health Service, by the Rosentiel Foundation, and by the Elsa D. Jameson Memorial Fund.

of the morphology of cells from the blood with those cells seen in tumor imprints, smears obtained from blood-vessel endothelium, normal epithelial tissues, and connective tissue aided materially in the identification of some cells. All blood smears were stained by the Wright-Giemsa technique.

Results

The incidence of "atypical" cells in 525 patients with cancer and 122 patients without cancer is shown in tables 1 to 3. Cells in the peripheral blood as well as cells in the blood draining tumor sites are included. Examination of the blood from a small group (10 subjects) of normal persons failed to reveal a significant number of "atypical" cells.

Figures 1 through 19 show some representative "atypical" cells seen in the blood of the subjects studied. In general, these cells can be divided into two categories. The cells which are usually seen in the blood or bone marrow belong to the first group. Even though these cells may not offer much difficulty to the experienced person, they are often incorrectly identified by technologists performing preliminary screening examinations. The first group of cells includes myeloblasts, large-sized promyelocytes, large plasma and plasma-like cells at various stages of maturation, macrophages, megakaryocytes, and, occasionally, an osteoclast.

Cells which are not usually seen in the blood or bone marrow belong to the second group. Most of these cells have been difficult to classify. It is possible that some cells in this group may actually be tumor cells, but, because they do not meet all the criteria for tumor cells, they have not been included among the "positive" smears (*1*, *2*). These criteria include the immature appearance of the cell with large nuclei and nucleoli, large size, staining characteristics, and resemblance to cells observed in smears from tumors. As a matter of fact, re-examination, by the senior author, of 100 slides labeled by our technologists as having "atypical" cells revealed 5 which contained tumor cells. The cells belonging to the second group have varied from easily identifiable endothelial, epithelial, or fibroblastic cells to cells of large size (more than 50μ), with either abundant, light-blue cytoplasm or deep-blue, scanty cytoplasm, and a large, darkly staining nucleus, occasionally containing a light-blue nucleolus in smears stained by the Wright-Giemsa technique.

The majority of difficult decisions regarding tumor cells in the blood is presented by the blood obtained from the veins draining the tumor sites of the gastrointestinal tract or the lung. In the former, the presence of immature lymphoid and plasma-cell elements may confuse the observer. We have been particularly impressed with the appearance of fairly large (25 to 50μ) cells with several nuclei, deep-blue cytoplasm, and, occasionally, a nucleolus in the blood draining the gastrointestinal tract. Whether these seemingly immature cells originate in the abundant lymphoid tissue in the gastrointestinal tract is a problem requiring further investigation.

In the blood draining the lungs, the variety of macrophages and megakaryocytes is impressive. Particularly confusing is a smear in which a

TABLE 1.—*"Atypical" cells in patients with malignant tumors*

Site	Total patients	Number of patients with "atypical" cells
Gastrointestinal tract	145	36
Lung	67	15
Breast	145	27
Female genital tract	18	2
Prostate	12	0
Genitourinary tract	11	2
Skin	10	1
Malignant melanoma	18	6
Lymphosarcoma	37	7
Ear, nose, and throat	21	3
Other	41	2
Total	525	101

TABLE 2.—*"Atypical" cells in patients with benign tumors*

Site	Total patients	Number of patients with "atypical" cells
Gastrointestinal tract	7	1
Breast	13	1
Female genital tract	5	1
Genitourinary tract	3	2
Skin	2	1
Other	10	1
Total	40	7

TABLE 3.—*"Atypical" cells in patients without tumors*

Site	Total patients	Number of patients with "atypical" cells
Lung	14	2
Gastrointestinal tract	32	5
Genitourinary tract	3	0
Breast	17	2
Female genital tract	2	0
Other	14	0
Total	82	9

substantial number of tumor cells is accompanied by a large number of macrophages. These may be immature cells undergoing division, or mature cells with bizarre shapes and degenerating forms. The latter are large-sized cells (30 to 50μ) with eccentric nuclei, vacuoles, or inclusion bodies in the cytoplasm. In addition, megakaryocytes may offer considerable difficulty in identification of cells in the blood draining the lungs.

The number and kind of "atypical" cells is likely to be increased in blood draining organs which have been handled or traumatized severely, especially the stomach, lung, spleen, and small bowel. In addition, the appearance of some "atypical" cells may give a clue as to the origin of the

blood. For example, macrophages loaded with black (carbon?) particles can frequently be seen in the blood draining the lung, especially after manipulation. What are in all probability epithelial cells have been seen in blood from organs, with or without tumors, after the organs were handled.

Discussion

The unqualified diagnosis of a single cell as malignant is fraught with danger. Indeed, many pathologists still accept the demonstration of isolated "malignant" cells as only a tentative evidence of cancer. As exfoliative cytology has been applied to each additional organ, the education of the cytologist must include a further background on the morphology of the different normal, degenerating, and abnormal cells that will be encountered. Subsequently, the identification of a cell as malignant depends not only on its inherent morphological characteristics, such as large size, multiple nucleoli, immature chromatin pattern, and staining characteristics, but also on its environmental origin. Furthermore, although the importance of staining characteristics is depreciated by many investigators, the confusion which ensues when specimens prepared with different fixation and staining techniques are presented is very enlightening. Any of several stains can be used, provided the microscopist is experienced. The classification of tumor cells in the buffy-coat smears is likewise dependent on their "abnormal" presence in the peripheral or regional veins, staining characteristics, cytological features, and similarity to cells of imprint preparations of the tumor in question. It would be folly to suggest that such evidence is other than indirect. In the future it will probably be possible to culture such cells and thus further establish their identity and viability. A few successful cultures of malignant cells from the blood have been accomplished in our laboratory (*4*).

Recently, several methods have been published for the isolation and detection of tumor cells in the blood (*1*, *5*, *6*). There appears to be wide interest in, and application of, these methods. Hence, the major purpose of the present communication has been to draw the attention of those interested in the study of the blood of patients with cancer, and particularly of those interested in the study of the presence and viability of tumor cells in the blood of these patients, to the large number of cellular elements which may confuse the observer. These confusing elements vary: They may be immature normal hematopoietic cells (plasma cells at various stages of differentiation, myeloblasts, promyelocytes, and cells in mitosis), cells of normal tissue origin (endothelial, normal epithelial cells appearing in the blood following manipulation during surgery, and cells released by the spleen), as well as cells with obscure identities, which offer the greatest difficulty in differentiation from tumor cells. We have been impressed with the presence of many immature and abnormal elements in the blood of veins draining tumor sites of the gastrointestinal tract and lung. Particularly striking has been the increase in plasma cells. An increase in plasma cells in the blood and bone marrow, in association

with cancer, has been reported previously by several observers (*7–9*). In concentrated smear preparations, this increase is prominent. Often, there are extremely immature plasma cells released into the blood of such patients, and these cells are characterized by their large size (15 to 25μ), large nucleoli, and slate-blue cytoplasm, when they are stained by the Wright-Giemsa technique. The cells, however, retain the appearance of being hematopoietic in origin.

The reasons for the increased number and kind of "atypical" and immature cells in the blood of cancer patients remain largely obscure. It is possible that the presence of cancer produces a severe "stress" on the total hematopoietic system to provide an abnormally large number of cells which may not be necessarily reflected in the appearance, cellularity, or morphology of the bone marrow. On the other hand, it is possible that some of these cells are a result of involvement of the hematopoietic centers by direct invasion from tumor tissue, mechanical distortion by such invasion, or perhaps through the influence of some hitherto unknown "toxins" from the tumor tissue.

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PLATE 58

FIGURE 1.—Plasma-like cell in blood of patient with cancer of the stomach. *Note* large size of cell and double nucleus. × 1,000

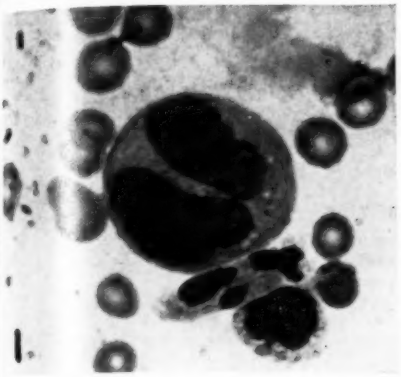
FIGURE 2.—Large macrophage (?) in blood draining cancer of the stomach after manipulation of the tumor. × 1,000

FIGURE 3.—"Atypical" cells in blood draining cancer of the colon after manipulation of the tumor. × 500

FIGURE 4.—Probable endothelial cells in blood draining carcinoma of the colon after handling of the tumor. × 320

FIGURE 5.—Blast cell (plasmablast or fibroblast) in blood of patient with cancer (primary site unknown). × 1,000

FIGURE 6.—"Atypical" cell in blood of a patient with a benign polyp of the rectum. × 500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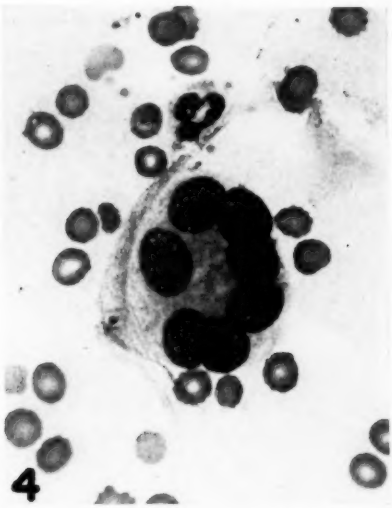
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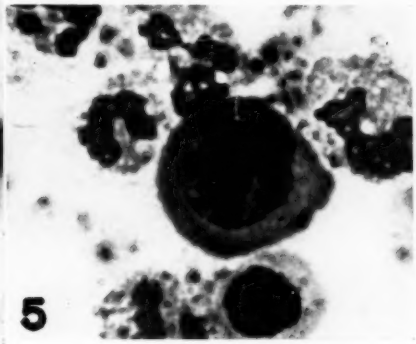
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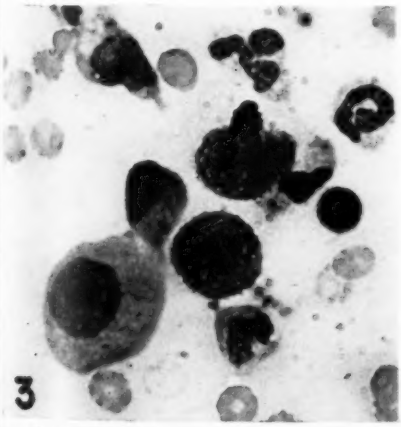
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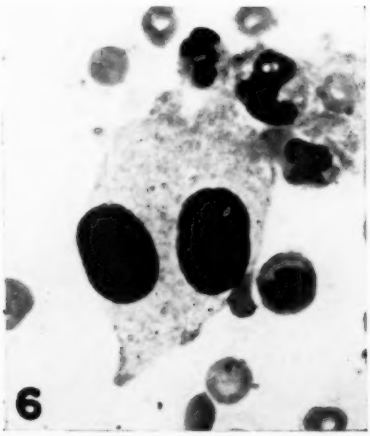
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PLATE 59

FIGURE 7.—Megakaryocyte in blood draining cancer of the lung after handling of the tumor. × 1,000

FIGURE 8.—A group of cancer cells accompanied by a macrophage from a patient with cancer of the lung. The blood was obtained from an arm vein after the tumor was manipulated during operation. × 320

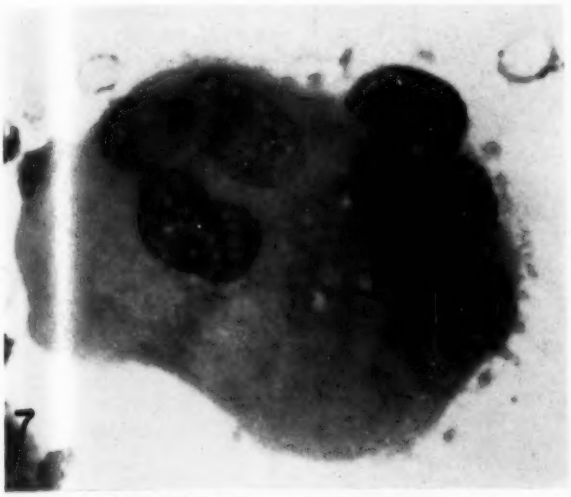
FIGURE 9.—"Atypical" cells from blood draining cancer of the colon after manipulation of the tumor. × 320

FIGURE 10.—Granulocytic cell in division in blood of patient with metastatic cancer (primary site unknown). × 1,000

FIGURE 11.—Macrophage in blood of patient with cancer of the lung. Particles probably represent carbon. × 320

FIGURE 12.—Macrophage from blood draining cancer of the head of the pancreas. × 320

FIGURE 13.—Probable promyelocyte in the blood of patient with cancer of the breast. × 800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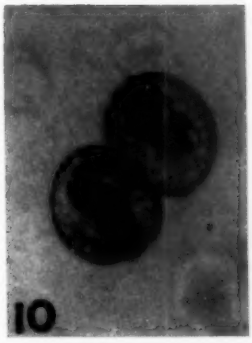
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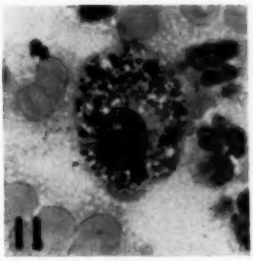
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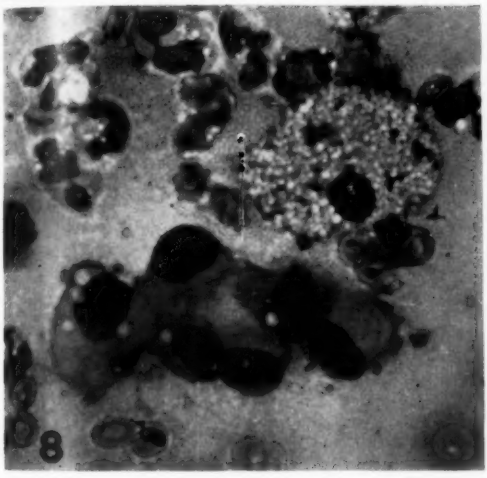
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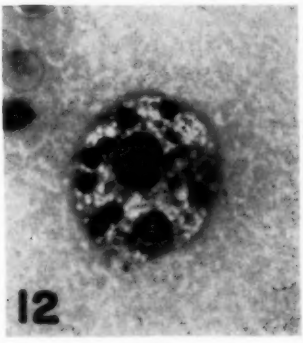
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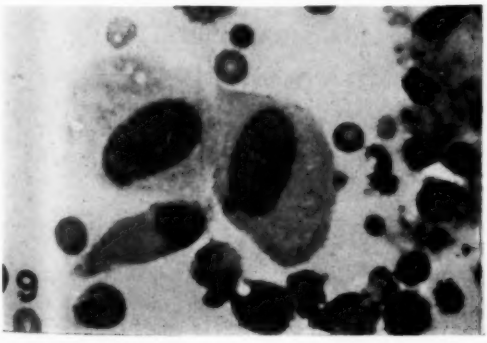
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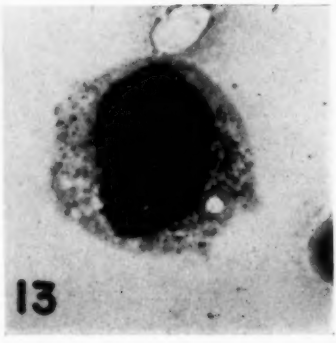
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PLATE 60

FIGURE 14.—Clump of "atypical" cells in peripheral blood of a patient with carcinoma of the rectum. Blood was obtained after surgery. × 500

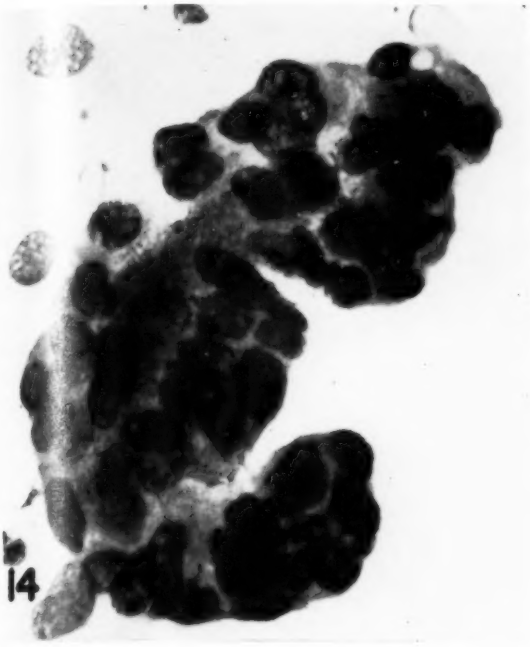
FIGURE 15.—Clump of nuclei with ill-defined cytoplasm in the blood of a patient with reticulum-cell sarcoma. The origin of the cell(s) is probably endothelial. × 1,000

FIGURE 16.—Large, double-nucleated cell from blood draining cancer of the lung after manipulation of the tumor. × 500

FIGURE 17.—Myelocyte in blood of a patient with cancer of the breast. × 1,000

FIGURE 18.—Macrophage in the blood of a patient with cancer of the lung. × 500

FIGURE 19.—Macrophage with an ingested large cell in blood of a patient with reticulum-cell sarcoma. × 1,000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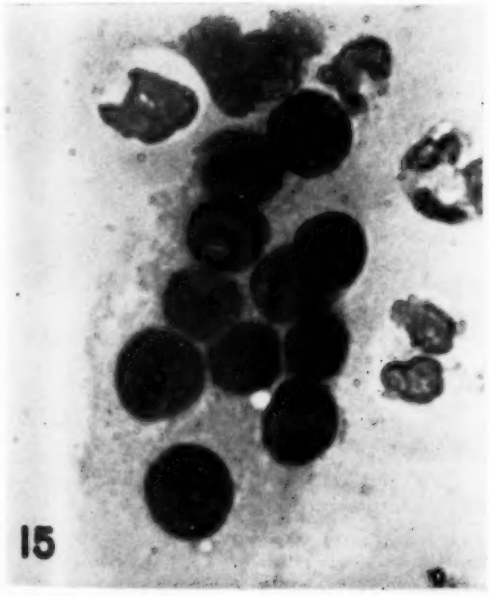
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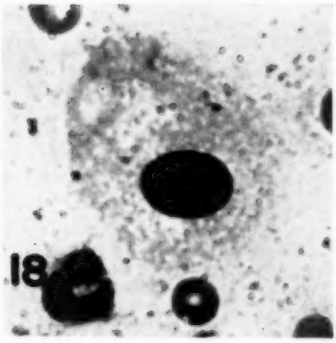
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Effects of Miracil D, Amodiaquin, and a Series of Other 10-Thiaxanthenones and 4-Aminoquinolines Against a Variety of Experimental Tumors *In Vitro* and *In Vivo*[1, 2]

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Summary

Miracil D, a 10-thiaxanthenone with pronounced activity as a schistosomicide, exhibits a wide spectrum of carcinostatic effectiveness against a variety of transplantable mouse tumors at nontoxic dosage levels. Sarcoma 180 and lymphoid leukemia L1210 ascites respond most readily, followed by Adenocarcinomas 755 and E0771; Glioma 26 and the Ehrlich ascites carcinoma show a marginal response at best. Oral administration of the drug appears preferable to intraperitoneal injection in some instances. Amodiaquin, a 4-aminoquinoline with potent antimalarial effects, has only borderline carcinostatic activity in the same tumor spectrum. The only tumor to respond significantly after oral administration of this agent is Adenocarcinoma 755. Seventeen other 10-thiaxanthenones and 12 other 4-aminoquinolines have been compared to these 2 compounds in terms of relative cytotoxicity in tissue cultures of human and mouse brain tumors, maximum-tolerated dose levels in the mouse, and carcinostatic activity against a variety of mouse tumors. None of the miracil analogues approaches the pronounced activity of miracil D *in vivo* and none of the amodiaquin analogues is superior to amodiaquin in this regard. In tissue culture, many of the 10-thiaxanthenones are more active than the parent compound; there is no correlation between activity *in vivo* and *in vitro* or between either of these indices and schistosomicidal effectiveness. About half the 4-aminoquinolines are as active as amodiaquin in the brain-tumor cultures, while the others are less active; again, there is little relationship between the effects *in vivo* and *in vitro* or between either of these and antimalarial activity.—**J. Nat. Cancer Inst. 22: 567–579, 1959.**

Several years ago, it was shown (*1*, *2*) that the potent antimalarial quinacrine and several other acridines exerted a powerful inhibitory effect on the growth of human and mouse brain tumors in tissue culture, but failed to affect a variety of transplantable tumors in the mouse. On the basis of comparative analytical data obtained in tumors exposed to

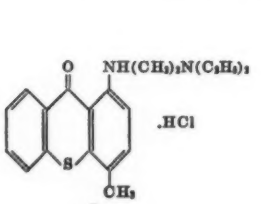
[1] Received for publication September 24, 1958.

[2] This work has been supported in part by U.S. Public Health Service research grant C-2332, the Charles Ulrick and Josephine Bay Gift for Research in Chemotherapy of Brain Tumors, and the Alma Toorock Memorial Fund. A preliminary report was presented at the International Cancer Congress in London, July 7, 1958.

[3] With the able assistance of Shirley Brindle, Ruth Eising, Annelies Herrmann, and Gloria Semente.

quinacrine *in vivo* and *in vitro*, it was postulated that this discrepancy was related to quantitative differences in drug concentration; the level of quinacrine attainable in the mouse tumors *in vivo*, without the appearance of frank signs of toxicity, approached but never equaled the minimum effective concentration *in vitro*.

This finding suggested a search for other types of heterocyclic compounds which might have pronounced activity in tissue culture but at the same time a more favorable distribution pattern and therapeutic index in the tumor-bearing mouse. The present report summarizes results obtained with a series of eighteen 10-thiaxanthenones including miracil D (Nilodin, Lucanthone) (I) and a group of thirteen 4-aminoquinolines related to amodiaquin (Camoquin®) (II).[4] Both of these



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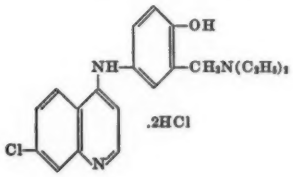


II

agents exhibit significant activity inother biological systems—amodiaquin primarily in malaria (*3–6*) and miracil D in schistosomiasis (*7–10*). The available literature does not contain any previous data on the effect of these 4-aminoquinolines on experimental neoplasms, but miracil D has been examined briefly in two earlier studies. Hackmann and associates (*11*) found it effective in retarding the growth of the Walker rat carcinosarcoma, less active against a benzopyrene-induced rat sarcoma, and entirely inactive against the solid form of the Ehrlich carcinoma in mice. Pirwitz and colleagues (*12*) were unable to demonstrate any effect of this agent against the Ehrlich ascites carcinoma.

Materials and Methods

In the present study, these 2 series of compounds were tested against human and mouse brain tumors *in vitro* and against 4 solid and 2 ascitic transplantable mouse tumors *in vivo*. The tissue-culture experiments utilized primary short-term explants of human glioblastoma multiforme, obtained at the time of surgical intervention,[5] or of mouse Glioma 26; the explants in Maximow slide lying-drop cultures were exposed to a nonsynthetic feeding fluid to which the compounds under test were added at various concentrations. The human and mouse brain tumors gave essentially the same results in these tests. The details of this

[4] Samples of all these compounds were generously made available by Parke, Davis & Company. We are indebted to Burroughs Wellcome Ltd. for an additional supply of miracil D.

[5] These samples were made available through the cooperation of Dr. Joseph Ransohoff and Dr. J. Lawrence Pool of the Department of Neurosurgery.

technique and of the damage rating employed to assess the effects of the compounds have been published (*1*, *13*, *14*).

For the evaluation of carcinostatic activity *in vivo*, the compounds were administered at the previously determined maximum-tolerated dose to mice of the appropriate strain bearing Sarcoma 180 (RIII), Adenocarcinoma 755 (C57BL), Adenocarcinoma E0771 (C57BL), Glioma 26 (C57BL), Ehrlich ascites carcinoma (RIII), and lymphoid leukemia L1210 ascites (DBA or DBA × Swiss hybrids). For the 4 solid tumors, daily intraperitoneal injections (except Sunday) were started on the day after tumor transplantation; for the Ehrlich ascites tumor, only 3 intraperitoneal injections were given, on the 1st, 2d, and 3d day after tumor-cell inoculation; for leukemia L1210, compounds were administered subcutaneously every day except Sunday. All solutions were prepared daily.

The effect on the solid tumors was assessed by a statistical comparison of the mean weights of tumors removed from groups of 20 control and 20 treated mice after 1 to 2 weeks of treatment; the effect on the ascites tumors was evaluated on the basis of the comparative survival times of groups of 10 or 20 treated and untreated animals. In most of the tests *in vivo*, a group of mice treated with a compound of known carcinostatic effectiveness was included as an added control.

In a small number of experiments, amodiaquin and miracil D were also administered by mouth to tumor-bearing mice. In these instances, the animals were given free access to ground mouse diet into which the compounds had been incorporated at levels designed to supply the maximum-tolerated oral dose.

Results and Discussion

Miracil D

In tissue culture, miracil D exhibited moderate activity, with a minimum inhibitory concentration of about 3×10^{-4} M (M/3000–M/4000). Under these conditions, this agent was only one tenth to one twelfth as active as quinacrine (*1*).

Table 1 summarizes the toxic effects of miracil D in normal mice at various dosage levels and by different routes of administration. The maximum-tolerated dose for repeated daily administration was found to be about 70 mg. per kg. intraperitoneally, about 225 mg. per kg. subcutaneously, and about 0.075 percent in the diet, estimated to yield an approximate average daily oral dose of 150 mg. per kg. Gross and microscopic examination of the heart, liver, spleen, kidney, small intestine, sternum, and brain of a small number of mice surviving 1 or 5 doses of miracil D at 150 to 200 mg. per kg. disclosed no clearly reproducible toxic lesions. These findings are in general agreement with the experience of other investigators (*9*, *11*, *15–17*). After the intraperitoneal administration of higher doses (250 or 300 mg./kg.), mice often exhibited a slight tremor shortly before death. This is of interest in view of the fact that cortical stimulation, manifested by hallucinations, paranoid reactions, and

TABLE 1.—*Toxicity of miracil D*

Route	Dose (*mg./kg./day*)	Number of doses	Dead/total	Percent body weight change
Intraperitoneal	500	1	3/3	
	300	1	3/3	
	200	1	2/3	
	100	1	0/3	
	300	5	5/5	
	250	5	5/5	
	200	5	1/5	−13
	150	5	1/5	−11
	100	5	3/10	−10
	80	5	1/5	−9
	70	5	0/5	−4
	60	5–7	1/50	−5
	50	5	1/10	−3
	40	5–17	6/325	−3
	25	5	0/5	+1
	20	6	0/20	−1
Subcutaneous	250	5	0/5	−10
	200	5	0/5	−6
	150	5	0/5	−2
Oral	0.5% in the diet		13/20	−30
	0.3% in the diet		1/20	−23
	0.2% in the diet		1/40	−15
	0.1% in the diet		2/100	−11

restlessness, have been observed in a few human subjects given 15 to 30 mg. per kg. per day for one or two 5-day periods.[6]

The effect of miracil D on 6 transplantable mouse tumors is presented in detail in table 2. The most responsive tumors were leukemia L1210 and Sarcoma 180; Adenocarcinomas 755 and E0771 also were affected, the latter particularly when the oral route of administration was used. The effect on the Ehrlich ascites carcinoma and on Glioma 26 was unimpressive. The effect of miracil D on Sarcoma 180 and Adenocarcinomas 755 and E0771 was not as pronounced as that of 6-mercaptopurine; in 10 representative experiments with these 3 tumors, the mean tumor weight after administration of this purine antagonist at 30 to 50 mg. per kg. per day was 15 percent of the control tumor weights (range 3–24%). The increase in survival time in mice bearing leukemia L1210 after treatment with miracil D was comparable to that produced by 5-fluorouracil (30 mg./kg./day) or 8-azaguanine (100–150 mg./kg./day) given by the same route of administration. In most of the experiments with solid tumors, significant carcinostasis, when observed, was achieved in the absence of measurable host toxicity. The results obtained with leukemia L1210 provided a satisfactory dose-response curve, since daily subcutaneous doses of 150 mg. per kg. more than doubled the prolongation of survival time observed at 60 mg. per kg. In 9 experiments not shown in the table, drug treatment by the intraperitoneal or oral route was begun on the 4th or 6th rather than the 1st day after transplantation. Under

[6] Clinical studies of miracil D have been initiated in a variety of neoplastic diseases (A. Gellhorn, J. E. Ultmann, and T. Zaidenweber, personal communication).

these conditions, miracil D no longer caused significant inhibition of the growth of the solid tumors.

In experimental cancer chemotherapy, many compounds are available which exhibit carcinostatic activity against one neoplasm or another. Relatively few of these agents, however, have a wide spectrum of activity against a variety of experimental tumors; these latter are, in general, of greater interest in clinical chemotherapy. The present results indicate that miracil D belongs to the more select group of compounds proposed for early clinical evaluation.[7]

Amodiaquin

This compound was quite active in tissue culture, with a minimum inhibitory concentration of about 5×10^{-5} M. In this regard, it was similar to quinacrine, whose pronounced activity *in vitro* first prompted the examination of this series of heterocyclic compounds.

The toxicity of various levels of amodiaquin is summarized in table 3. Comparison of these data with the analogous findings with miracil D indicates that the 4-aminoquinoline more frequently causes death of the mice without appreciable previous weight loss, whereas the major effect of the 10-thiaxanthenone at intermediate toxic levels is on body weight.

Table 4 presents the detailed results of the evaluation of amodiaquin in 6 transplantable mouse tumors. With the exception of Adenocarcinoma 755, which was strongly inhibited by oral administration of the drug, none of the tumors was affected in a significant and consistent manner. In a series of patients with glioblastoma multiforme, amodiaquin exerted no beneficial clinical effect.[8]

Other 10-Thiaxanthenones

The structures, tissue-culture activities, maximum-tolerated doses, and carcinostatic activities *in vivo* of 17 other 10-thiaxanthenones (*cf. 18*) are summarized in table 5. These comparative studies indicate, on the one hand, that none of the miracil derivatives retains the spectrum of carcinostatic activity against these mouse tumors which is shown by the parent compound, although scattered positive results were obtained. Eleven of them, on the other hand, are endowed with greater relative cytotoxicity against the human and/or mouse brain tumors in tissue culture. The failure of this system *in vitro* to correlate with the results *in vivo* has been emphasized and discussed recently (*14*). It may be mentioned in passing that there is no correlation between these tissue-culture or mouse-tumor data and the relative schistosomicidal potency of these compounds in mice.

Attempts to elucidate structure-activity relationships in this series of 10-thiaxanthenones with reference to their cytotoxicity *in vitro* have not been particularly fruitful. It is interesting to note, however, that all the highly active compounds contain a basic diamine side-chain. On the

[7] *See* footnote 6.

[8] A. Gellhorn and H. G. Thompson, unpublished results.

TABLE 2.—*Effect of miracil D on various mouse tumors*

Tumor	Dose*	Body weight change (*control/treated*)	Deaths (*treated*)	Mean tumor weights (*mg.*) or survival times (*days*) (*control/treated*)	D/S_D †
Sarcoma 180	6 × 20	−1/−1	0	320/249	1. 7
	6 × 40	−3/−2	1	446/305	2. 6
	6 × 40	−3/−5	0	446/304	2. 9
	6 × 40	−1/−4	0	320/191	3. 2
	6 × 40	+12/+2	0	297/161	3. 2
	9 × 40	+1/−1	1	334/163	2. 9
	6 × 60	−1/−4	0	320/178	3. 2
	8 × 0. 1%	−9/−16	0	362/240	2. 6
	8 × 0. 2%	−9/−22	0	362/168	3. 8
Adenocarcinoma 755	6 × 40	−6/−9	0	563/499	—
	7 × 40	+2/−1	0	443/477	—
	8 × 40	−15/−8	0	1005/397	6. 1
	11 × 40	+7/−1	0	550/163	3. 9
	12 × 40	+11/−1	0	620/141	3. 2
	13 × 40	0/−6	0	479/160	4. 0
	13 × 150‡	0/−13	0	479/234	2. 5
	21 × 0. 05%	−7/−13	1	408/242	2. 0
	21 × 0. 075%	−7/−14	5	408/321	0. 8
	8 × 0. 1%	−4/−10	0	263/159	3. 6
	14 × 0. 1%	+4/−6	0	629/224	4. 3
	8 × 0. 2%	−4/−15	0	263/91	6. 4
Adenocarcinoma E0771	6 × 40	+2/−1	0	344/257	—
	7 × 40	+6/0	0	334/277	1. 2
	7 × 40	+2/−8	0	463/353	1. 9
	8 × 40	+12/+9	2	575/410	2. 1
	10 × 40	−4/−12	0	470/276	3. 3
	7 × 60	+2/−7	1	463/241	4. 5
	10 × 150‡	−4/−11	0	470/219	4. 6
	8 × 0. 1%	−3/−8	0	329/136	5. 1
	8 × 0. 2%	−3/−15	1	329/95	6. 7

Glioma 26	10 × 40	+4/−5	0	364/261	1. 1
	21 × 0. 05%	−2/−6	0	973/597	1. 6
	21 × 0. 075%	−2/−6	0	973/443	2. 5
	15 × 0. 1%	−2/−14	2	435/288	1. 0
	15 × 0. 2%	−2/−20	0	435/85	2. 6
Ehrlich ascites carcinoma	3 × 40			11. 3/13. 2	1. 9
	3 × 40			9. 6/12. 4	4. 4
	3 × 40			12. 4/12. 6	—
	3 × 40			20. 3/23. 6	0. 9
	3 × 50			10. 1/13. 8	2. 6
	3 × 50			14. 9/18. 1	2. 3
	3 × 60			13. 6/17. 0	2. 0
	3 × 60			20. 3/25. 5	0. 9
	3 × 80			20. 3/25. 1	1. 6
	3 × 100			20. 3/28. 6	2. 5
	15 × 0. 1%			15. 2/14. 6	—
Leukemia L1210 ascites	30 daily			9. 8/10. 4	0. 8
	45 "			9. 8/10. 3	0. 5
	60 "			9. 8/13. 9	3. 1
	60 "			7. 2/9. 3	4. 1
	60 "			8. 3/10. 6	2. 1
	60 "			7. 1/8. 3	3. 5
	60 "			10. 1/14. 1	2. 9
	75 "			9. 8/14. 5	5. 2
	90 "			9. 8/14. 3	6. 7
	120 "			10. 2/15. 5	6. 5
	150 "			8. 7/14. 7	11. 6

*Number of injections × mg. per kg. per day or number of days on diet containing the stated percentage of the drug.

†$D/S_D = \frac{M_1 - M_2}{\sqrt{\sigma_{m1}^2 + \sigma_m^2}}$. If this value is 2.5 or larger, the difference between the means is clearly significant ($P<0.01$).

‡Subcutaneous.

TABLE 3.—*Toxicity of amodiaquin*

Route	Dose (*mg./kg./day*)	Number of doses	Dead/total	Percent body weight change
Intraperitoneal.....	500	1	3/3	
	250	1	3/3	
	125	8–12	15/80	0
	100	7–17	19/220	+1
	50	5	0/3	−5
Oral.....	0.6% in the diet		2/60	−8
	0.5% in the diet		1/40	−10
	0.4% in the diet		0/20	−3
	0.25% in the diet		0/20	−6
	0.15% in the diet		1/20	+6

premise that solubility might be an important factor in the activity *in vitro*, the solubility of 14 of the thiaxanthenones in water at *p*H 7.0 was determined.[9] Appraisal of these data indicates that there is no general correlation in this series between solubility at *p*H 7.0 and relative activities in tissue culture. The biological data suggest that the presence of a chlorine atom in the 7-position favors the inhibitory action of these compounds on the brain-tumor cultures, but a precise definition of the significance of various other structural elements has not yet been possible. An additional series of 10-thiaxanthenones is currently under investigation to provide further information which may be useful in elucidating structure-activity relationships in this series.

Other 4-Aminoquinolines

Table 6 presents, in the same form as table 5, the pertinent data on 12 other 4-aminoquinolines. Several compounds inhibited the growth of one or another of these mouse tumors, but none of these amodiaquin congeners was superior to the parent compound. *In vitro*, the replacement of the chlorine atom in the 7-position by a methyl, methoxy, or nitro group yielded compounds of about equivalent relative cytotoxicity; alterations in the composition of the side-chain had variable effects. As in the thiaxanthenone series, correlation of results *in vitro* with carcinostatic activity *in vivo* was poor. The tissue-culture data also bear little direct relationship to the antimalarial potency of the various derivatives (*cf. 3*).

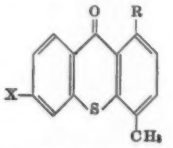
[9] The authors are indebted to Dr. J. M. Vandenbelt and associates of Parke, Davis & Company for the solubility determinations.

TABLE 4.—*Effects of amodiaquin on various mouse tumors*

Tumor	Dose*	Body weight change (*control/treated*)	Deaths (*treated*)	Mean tumor weights (*mg.*) or survival times (*days*) (*control/treated*)	D/S_D†
Sarcoma 180	7 × 100	+5/+1	2	829/779	—
	8 × 100	+6/−4	2	250/146	2. 5
	8 × 125	+2/−6	7	686/578	—
	9 × 0. 5%	+5/−3	0	138/123	—
Adenocarcinoma 755	6 × 100	+1/−3	0	589/490	1. 3
	8 × 100	+13/+1	3	315/156	2. 1
	8 × 100	+8/+6	0	602/307	3. 7
	12 × 125	+21/+11	1	1090/910	1. 5
	13 × 0. 6%	+12/−9	1	623/101	8. 0
	14 × 0. 6%	+18/−2	0	1726/564	4. 7
Adenocarcinoma E0771	7 × 100	+3/−3	1	486/551	—
	8 × 100	+24/+14	5	1153/656	2. 6
	8 × 100	+14/+4	0	797/622	1. 0
Glioma 26	10 × 100	0/+4	5	871/849	—
	11 × 100	−1/−3	1	260/253	—
	8 × 125	−2/−12	2	631/196	2. 9
	12 × 125	+15/+7	5	830/401	2. 2
	12 × 0. 15%	+13/+6	1	635/735	—
	12 × 0. 25%	+13/−6	0	635/656	—
	14 × 0. 4%	+19/−3	0	731/616	0. 7
	13 × 0. 5%	−1/−16	1	260/203	0. 9
	14 × 0. 6%	+19/−14	1	731/347	2. 6
Ehrlich ascites carcinoma	3 × 100			9. 7/8. 4	—
	3 × 100			13. 2/14. 8	1. 0
	3 × 125			12. 6/11. 7	—
Leukemia L1210 ascites	125 daily			6. 4/5. 9	—
	150 daily			7. 2/6. 6	—

* † *See* corresponding footnotes to table 2.

TABLE 5.—*Tissue-culture cytotoxicity, mouse toxicity, and carcinostatic activity of miracil analogues*



-R	X	*In vitro**		MTD† (*mg./kg.*)	Activity *in vivo* against‡					
		Human	Mouse		S180	755	E0771	Gl 26	Ehrlich	L1210
$-NCH_3(CH_2)_2N(C_2H_5)_2$	Cl	50	30	20	0/1	0/1	0/1	0/1	0/2	1/2
$-NCH_3(CH_2)_2N(C_2H_5)_2$	Cl	50	20	20	0/1	0/1	0/1		0/2	0/2
$-NCH_3(CH_2)_2N(CH_3)_2$	Cl	50		50	1/3	1/2	0/2	0/1	0/2	1/3
$-NCH_3(CH_2)_2NC_2H_5(CH_2)_2OH$	Cl	25		30	0/1	0/1	0/1		0/3	0/3
$-NH(CH_2)_3N(CH_3)_2$	H	25		60	2/3		0/2	0/1	0/1	0/2
$-NCH_3(CH_2)_2N[(CH_2)_2OH]_2$	Cl	15		30					0/1	
$-NHCH_2CHOHCH_2N(C_2H_5)_2$	H	10		25	0/2	1/2	0/2	0/1	0/3	2/4
$-NCH_3(CH_2)_2N(C_2H_5)_2$	H	10		10	0/2	1/2	0/2	0/1	0/3	0/3
$-NH(CH_2)_3N[(CH_2)_2OH]_2$	H	10		25	1/3	0/1	0/1	0/1	0/1	0/2
$-NCH_3(CH_2)_2N(CH_2)_5$	Cl	10		25	0/1	0/1	0/1		0/2	0/2
$-NCH_3CHCH_3CH_2N(CH_3)_2$	H	10		25	0/1	0/1	0/1		0/1	0/2
$-NH(CH_2)_2N(C_2H_5)_2$ *miracil D*	H	3	4	60	6/6	7/9	5/9	1/4	3/11	8/9
$-NCH_3(CH_2)_2NC_2H_5CH_2COH(CH_3)_2$	Cl	4		75	0/1		0/1	0/1		
$-NCH_3(CH_2)_2N[(CH_2)_3CH_3]_2$	Cl	4		50	0/1	1/1	0/2		0/2	0/2
$-NC_2H_5(CH_2)_2OH$	H	1		100	0/1	0/1	0/1	0/1	0/2	0/3
$-NCH_3(CH_2)_2OH$	H	1		30	0/1	0/1	0/2		0/2	0/2
-Cl	Cl		0	30	0/1	0/1	0/1	0/1	0/1	0/1
$-NCH_3(CH_2)_2Br$	H		0							

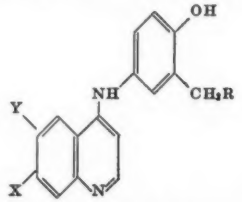
*The activity *in vitro* is expressed in terms of relative values, assigning a figure of 1 to a minimum inhibitory concentration (MIC) of M/1000, a figure of 10 to a MIC of M/10,000, and so on. A figure of 0 indicates that the MIC is greater than M/1000.

†MTD = maximum-tolerated dose in mice given 1 injection on each of 5 consecutive days.

‡These results are summarized in terms of the number of experiments with significant inhibition ($D/S_D = 2.5$ or greater) without prohibitive host toxicity over the total number of experiments.

TABLE 6.—*Tissue-culture cytotoxicity, mouse toxicity, and carcinostatic activity of amodiaquin analogues*

4-(4-Quinolylamino)-α-amino-o-cresols

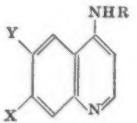


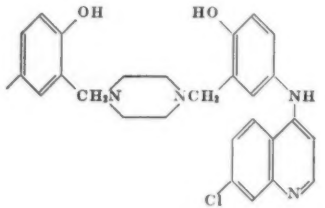
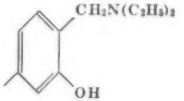
-R	X	Y	*In vitro**		MTD† (*mg./kg.*)	Activity *in vivo* against‡					
			Human	Mouse		S180	755	E0771	Gl26	Ehrlich	L1210
$-N(C_2H_5)_2$	CH_3	H	50	20	75	0/1	1/2	0/1	0/1	0/1	
$-NHCH_2CH(CH_3)_2$	Cl	H	30	30	30	0/3	1/3	1/1	0/1	0/3	0/1
$-N(C_2H_5)_2$	OCH_3	H	20	30							
$-N(C_2H_5)_2$	NO_2	H	30	20	75	0/1		0/1		0/1	
$-N(CH_2)_4$ *Amopyroquin*	Cl	H	15	25	100	1/3	0/2	0/2		0/1	
$-N(C_2H_5)_2$ *Amodiaquin*	Cl	H	20	20	125	1/4	3/6	1/3	1/8	0/3	0/2
$-N(C_2H_5)_2$	Cl	Cl	4		100	0/2	2/2	0/1	0/2	0/4	
$-N[(CH_2)_3]_2O$	Cl	H	2		75	1/3	0/2			0/1	
$-N[(CH_2)_3CH_3]_2$	Cl	H	1		50	0/2	1/2	1/2		1/1	0/1

* † ‡ *See* corresponding footnotes to table 5.

TABLE 6.—*Tissue-culture cytotoxicity, mouse toxicity, and carcinostatic activity of amodiaquin analogues*—Continued

Miscellaneous 4-aminoquinolines



-R	X	Y	*In vitro**		MTD† (*mg./kg.*)	Activity *in vivo* against‡					
			Human	Mouse		S180	755	E0771	G126	Ehrlich	L1210
	Cl	H	10		30	0/1	0/1	0/1		0/1	
	Cl	H	10		100	0/1	0/1			0/1	
-H	Cl	H	3		40	0/1	0/1			0/1	
$-NH_2$	Cl	H	0	0	30	0/2	0/1	0/1		0/1	

* † ‡ *See* corresponding footnotes to table 5.

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Transplantation of Carbon Tetrachloride-Induced Hepatomas in Mice [1, 2]

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Summary

Five carbon tetrachloride-induced hepatomas from a mouse that was killed 8 months after the last administration of CCl_4 were successfully transplanted to mice of the same strain, but transplantations of primary tumors from mice whose CCl_4 treatments ceased after 11 weeks or less were not successful. This suggests that there is a progressive increase in the capacity for autonomous growth in the primary hepatoma. One of the CCl_4-induced hepatomas maintained by serial, intrasplenic transplantations is now in its fifteenth generation. During passage through successive hosts there have been only slight morphological changes, but a gradual decrease in time from 4 months to 2 months between transplantations has occurred.—J. Nat. Cancer Inst. 22: 581–591, 1959.

Hepatomas may be induced in mice by repeated oral administration of carbon tetrachloride (*1*, *2*). A high incidence of tumors, 88.2 percent, has been reported in mice of 4 inbred strains that received from 23 to 58 doses of 0.04 ml. of CCl_4 (*3*). The incidence varies both with the total amount that is administered and with the total time, *i.e.*, spacing of doses, during which a given amount is administered (*4*). The mechanism by which CCl_4 induces the development of hepatomas is not known (*3*). Obvious liver necrosis and accompanying regenerative activity are not necessary for hepatoma induction but, as pointed out by Eschenbrenner and Miller (*5*), this does not rule out the possibility that non-necrotizing doses of CCl_4 may produce a chronic regenerative or hyperplastic state of the liver.

Edwards and Dalton (*3*) reported the successful subcutaneous transplantation of 1 out of 8 CCl_4-induced hepatomas, which was carried for at least 4 generations. Andervont and Dunn (*6*) found that 8 CCl_4-induced hepatomas failed to grow when transplanted, but later (*7*), of 30 such hepatomas, all except 2 grew in new hosts. Successful transplantation is frequently considered a criterion of neoplasia. In this labora-

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tory, numerous failures to establish a transplantable CCl_4-induced hepatoma at first seemed to support the idea that, if transplantability is a criterion, the nodules might be hyperplastic but not neoplastic. Subsequently, however, several such hepatomas were successfully transplanted from a host that was allowed to live for a long period after CCl_4 administration ceased.

Material and Methods

All mice used in this study were males of the BUB strain developed in this laboratory. No spontaneous hepatomas have been found in this strain which is now in its 40th generation. The mice were maintained at 78° F. on Purina laboratory chow and water *ad libitum*.

Carbon tetrachloride was administered by stomach tube (*8*) in doses of 0.1 ml. of a 40 percent solution in olive oil, *i.e.*, 0.04 ml. of CCl_4 per treatment, given 3 times per week for a total of 45 to 66 doses (*1–3*). The incidence of hepatomas induced by CCl_4 in the BUB strain is high, but the numerical incidence has not been determined because of the relatively small numbers of animals employed.

In each group of mice treated with CCl_4, about one third of the animals was given 3 daily intravenous injections of 0.2 ml. of thorotrast before CCl_4 administrations were started and X-ray pictures were taken at 2- to 4-week intervals. Control mice received only thorotrast. The radiopaque thorotrast is distributed throughout the reticuloendothelial system of the liver where it remains for long periods, and a small amount also enters the hepatic parenchymal cells where it is secreted into the bile and is no longer microscopically visible 80 days after administration (*9*). It is not included in the hepatomas as they form; hence, the position, number, and size of developing hepatomas can be detected in X-ray pictures (*10*). No hepatomas developed in our 20 control mice examined 192 to 514 days after injection of thorotrast only, but hepatomas did occur in CCl_4-treated BUB mice that were free of thorotrast. Therefore, we have assumed that thorotrast did not play an important part in the development and transplantability of the hepatomas described here. It must be pointed out, however, that thorotrast has been involved in tumor production, especially in vascular tumors of the liver and other organs (*11–13*), and Kuppfer cell tumors (*14*). Guimaraes and Lamerton (*12*) reported hepatoma development in thorotrast-treated mice, but hepatomas also occurred in some of their untreated control animals, so that the possible interactions between thorotrast and other concomitant, and possibly latent, diseases or forms of injury must be studied further.

The first-generation tumor transplants were made subcutaneously in the axillary region by trocar. Subsequently, both subcutaneous and intrasplenic transplants were made by injecting, by means of a syringe and 18-gauge needle, 0.02 to 0.05 ml. of minced tissue moistened with sterile 0.85 percent saline. Under light ether anesthesia, implants of tumor into the spleen were made by way of an incision through the dorsal body wall. The spleens were examined periodically by laparotomy.

The tumors were routinely fixed in Bouin's fluid and stained with Delafield's hematoxylin and eosin. Other cytological methods employed to compare the tumor cells with their cells of origin include Regaud's iron hematoxylin method for mitochondria (*15*), McManus' (*16*) periodic acid-Schiff method for glycogen, Sudan black B solution, saturated in 70 percent ethanol, for 1 minute on formalin-fixed frozen sections for fat, and methylene blue, *p*H 5.3, for basophilic ergastoplasm (*17*).

The mitotic index, *i.e.*, the number of mitotic figures per 1,000 cells, was determined in 5μ sections fixed in Bouin's fluid and stained with hematoxylin and eosin by counting the numbers of nuclei, mitotic figures, and binucleate cells in 100 miscroscope fields at a magnification of 1,125×. The number of binucleate cells was subtracted from the number of nuclei to obtain the number of cells. This was done because it is simpler to count nuclei than whole cells. The area ratios of nuclei to cytoplasm were determined by Chalkley's (*18*) method of recording the number of "hits" on nuclei and cytoplasm of 6 random points in the same 100 fields in which the above counts were made.

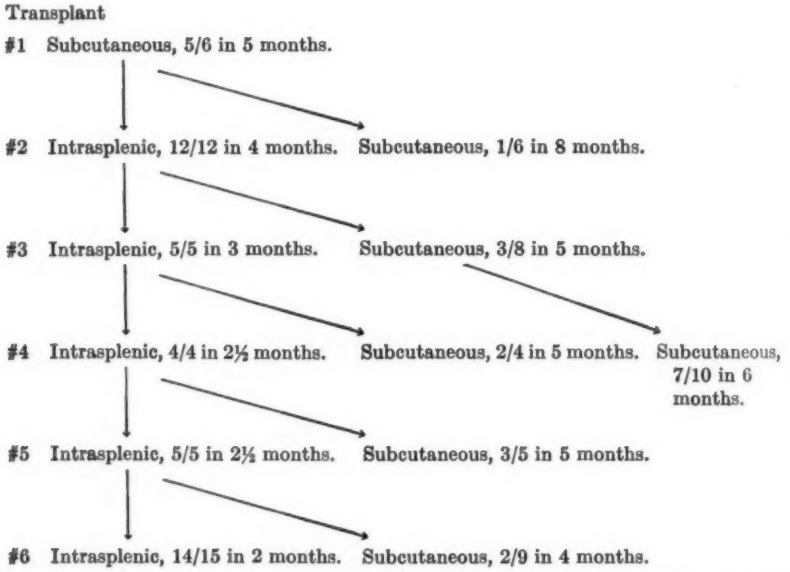
Observations

The primary hepatomas in the observations reported here were obtained from 3 mice that had developed tumors after receiving repeated oral administrations of CCl_4. In each group of mice so treated, some had previously been injected with thorotrast, and their CCl_4 treatments ceased as soon as X-ray pictures revealed developing hepatomas. Among the mice that had not received thorotrast, 1 was given 50 administrations of CCl_4 and killed 12 days after the last dose. Its liver was characteristically cirrhotic and studded with small nodules, like that in figure 4; 1 large nodule 10-mm. in diameter was presumed to be a hepatoma. This hepatoma was transplanted subcutaneously into 7 young mice of the same strain. No tumors developed during a 13½-month observation period. A second mouse, which had received 52 doses of CCl_4 was allowed to live for 11 weeks before it was killed. Nearly all traces of cirrhosis had disappeared but there was 1 nodule, 7 mm. in diameter. This nodule was transplanted subcutaneously into 9 recipients, and there were no takes in 12 months. We should point out here that numerous earlier attempts to transplant CCl_4-induced tumors always had been made within a few days after the last dose of CCl_4, and no takes were obtained.

Carbon tetrachloride administration to one thorotrast-injected mouse ceased after the 45th dose, because hepatomas which were thorotrast free were detectable in X-ray pictures of the liver. The animal was killed 8 months after the last CCl_4 administration. The cirrhosis associated with repeated CCl_4 poisoning had disappeared, but there were 9 well-defined hepatomas, 10 to 15 mm. in size. Five of these hepatomas were transplanted subcutaneously into a total of 31 young mice. The first of these 5 hepatomas to take, primary tumor #1, was palpable in 2 out of 6 recipients in 3 months, 3/6 in 4 months, and 5/6 in 5 months. Successful takes of the other 4 hepatomas were as follows: primary tumor #2: 1/5 in

6 months and 2/5 after 7 months; primary tumor #3: 1/7 in 6 months, 4/7 in 7 months, 5/7 in 9 months; primary tumor #4: 1/7 after 7 months; primary tumor #5: 3/6 after 9 months. Through 12 months, no takes were observed in the remaining mice. Cytologically the primary hepatomas differed principally in the degree of vacuolization of the cytoplasm, that is, in the relative amount of glycogen and fat, and in cell and nuclear size. These differences did not appear to be related to the incidence or rapidity of takes of the transplants. Most of the subcutaneous transplants reached 5 to 10 mm. in size 1 to 2 months after they became palpable, though a few grew more slowly. One originating from 4 of the 5 primary hepatomas was again transplanted, this time both subcutaneously and intrasplenically. Serial passages of all 4 tumors were maintained for 3 or 4 transplant generations, after which all except one were abandoned because all exhibited similar cytological and growth characteristics.

The one transplantable CCl_4-induced hepatoma which has been maintained, called Hepatoma BC3, is now in its fifteenth generation. It originated from the first hepatoma to take, primary hepatoma #1; the second transplant was made 4 months after the first. Subsequent takes of this tumor are shown in text-figure 1. From the second through the sixth transplant generations, there was a higher incidence of takes and more rapid growth of the hepatoma when it was implanted intrasplenically than when it was implanted subcutaneously. In both sites there was a gradual reduction in the intervals between transplantations, that is, the tumor reached transplantable size more and more rapidly during succes-


Transplant
#1 Subcutaneous, 5/6 in 5 months.
#2 Intrasplenic, 12/12 in 4 months.
Subcutaneous, 1/6 in 8 months.
#3 Intrasplenic, 5/5 in 3 months.
Subcutaneous, 3/8 in 5 months.
#4 Intrasplenic, 4/4 in 2½ months.
Subcutaneous, 2/4 in 5 months.
Subcutaneous, 7/10 in 6 months.
#5 Intrasplenic, 5/5 in 2½ months.
Subcutaneous, 3/5 in 5 months.
#6 Intrasplenic, 14/15 in 2 months.
Subcutaneous, 2/9 in 4 months.

TEXT-FIGURE 1.—Summary diagram of the history of the first through the sixth transplant generations of Hepatoma BC3.

sive passages. Since the seventh transplantation, the tumor has been maintained only intrasplenically. At present there is a latent period of approximately 4 weeks, and the tumor is transplanted 3 to 4 weeks later.

The mitotic index of primary tumor # 1 was very high: 23.3 mitotic figures per 1,000 cells as compared with 1.6 in the normal liver of a 90-day-old mouse (table 1). The mitotic indexes of the donor hepatomas in the second, eighth, and fourteenth transplant generations (table 1) were high but revealed no particular trend; however, at the time of transplantation no attempt was made to select a particular time of day to coincide with a possible diurnal rhythm (*19*).

TABLE 1.—*Mitotic indexes of primary and donor hepatomas*

	Nuclear density*	Binucleate cells*	Mitotic index†	Nucleocytoplasmic ratio‡ (*percent*)
Control liver	2141	345	1.6	6.1
Primary hepatoma #1	2807	105	23.3	12.4
2nd transplant	3315	93	7.7	11.8
8th transplant	4038	76	14.1	11.9
14th transplant	4168	18	11.0	13.9

*Number of nuclei and binucleate cells counted in 100 fields at 1,125×.
†Number of mitotic figures of all stages per 1,000 cells.
‡Ratio of nuclear area to cytoplasmic area as determined by the Chalkley (*18*) method.

As compared with the normal liver of an adult mouse, the number of nuclei per 100 microscope fields (table 1, nuclear density) was somewhat higher in the primary tumor, and gradually increased during serial passages of the tumor. Concomitantly, the number of binucleate cells gradually decreased. The ratio of nuclear area to cytoplasmic area in the primary tumor is approximately twice that in a normal liver (table 1). This is the result of a relative decrease in amount of cytoplasm per cell, and possibly also the result of an over-all increase in nuclear size (figs. 1 and 2). We have not attempted to measure the nuclei because the material was not critically prepared for this purpose. Nevertheless, variations in size are apparent. Clusters of cells sometimes differ from the surrounding ones in this respect, and it is not unusual to find occasional unusually large cells (fig. 6) in the primary hepatoma and all subsequent transplants. The nucleocytoplasmic ratios of the second, eighth, and fourteenth transplants varied little from that of the primary tumor (table 1), but, since the nuclear density gradually increased, there must have been a slight reduction in cell and nuclear size. By visual examination, the over-all nuclear size of the fourteenth generation transplant does appear to be smaller than that of the primary tumor (figs. 2 and 3). These slight variations do not involve a change in the essential character of the tumor and are, therefore, in sharp contrast to some of the dramatic changes in cell type and arrangement mentioned by Andervont and Dunn (*6*).

The hepatoma cells resemble their cells of origin, the hepatic parenchymal cells, in their organization into cords or sheets (fig. 5), between

which are blood vessels resembling sinusoids. A lobular pattern is lacking. Well-defined bile canaliculi with their associated terminal bars are frequently seen between contiguous hepatoma cells (fig. 6). We have not yet determined whether substances can be secreted into the canaliculi, but a marked distention of some of them suggests that materials may accumulate within them. There are no bile ducts or cholangioles into which they can drain. The nuclei are vesicular and contain numerous nucleoli, as do the hepatic parenchymal cells (figs. 1, 2, and 3). The hepatoma contains glycogen and sudanophilic lipide. The latter, in particular, is especially abundant, and many cells are distended, with large globules of fat (figs. 3 and 5) even when there is very little fat histochemically demonstrable in the liver of the host. The amount of glycogen and fat varies from cell to cell, however, from large accumulations to none (fig. 5). The basophilic ergastoplasm, which is aggregated into masses in liver cells, is uniformly dispersed throughout the cytoplasm of all hepatoma cells. The mitochondria are filamentous (fig. 6) as in the tumors described by Dalton and Edwards (*20*). Necrosis is rarely found in intrasplenic transplants of this tumor, even in nodules of such large size as 25 mm. in diameter. Splenic cells are, for the most part, excluded from the hepatoma, though a few may occasionally be found.

Transplantable Hepatoma BC3 remains contained within the capsule of the spleen, though it may reach as much as 25 mm. in diameter in mice that survive 3 months after implantation of the tumor. It does not appear to be invasive. We have sought evidence of metastasis but have found neither emboli nor isolated tumor cells in sections of the portal vein and in branches of the portal vein within the liver. Metastases to other organs have not been detected.

Discussion

Many important considerations concerning the transplantation of spontaneous and induced hepatomas in the mouse have been reviewed and critically discussed by Andervont and Dunn (*6*). It is the purpose of this report, in part, to discuss further some of their suggestions, on the basis of our experience with CCl_4-induced hepatomas. In our laboratory the CCl_4-induced hepatomas that were successfully transplanted differed from those that did not grow in new hosts in that a longer period elapsed between CCl_4 administration and tumor transplantation. The 5 successful transplants were obtained from a single host killed 8 months after the last treatment of CCl_4, whereas those that did not take were transplanted 11 weeks or less after the last treatment. The CCl_4-induced hepatoma which was successfully transplanted by Edwards and Dalton (*3*) was implanted in new hosts 6 months after the last administration of CCl_4. Andervont and Dunn (*7*) did not state the exact intervals between tumor induction and transplantation in their 28 successful transplants, but the possible intervals appeared to be between 2 and 8 months after the last injection of CCl_4. Chronic CCl_4 injury to the liver induces development of both hyperplastic nodules and hepatomas, and the livers of our CCl_4-

treated mice were conspicuously cirrhotic with numerous hyperplastic nodules. The nodules selected as tumors differed from the rest principally in size; histologically there was little, if any, difference. Andervont and Dunn (*6*) suggested that the hepatomas begin as a hyperplastic process which later develops into a true neoplasia. If this suggestion is correct, it is possible that the "hepatomas" which we transplanted 11 weeks or less after CCl_4 treatment were not far removed from the hyperplastic state. Thus, their growth may have been dependent on the particular conditions in the animals in which they arose—conditions which were lacking in the normal mice that were recipients of the tumor implants.

After CCl_4 treatment ceases, a relatively normal liver architecture is eventually re-established, with the disappearance of the hyperplastic nodules, but the true hepatomas persist and increase in size. Thus, in the true hepatomas there has been a loss of dependency on the host for growth control. The observation that the primary hepatomas were more readily transplanted when they had had a long sojourn in the host may suggest that time was necessary for the neoplastic change to occur and that this change is a gradual rather than a sudden one (*21*). This might be considered as representing a form of progression in the primary tumor (*22*). On the other hand, the maintenance of the host, under conditions in which the hyperplastic nodules disappear, is a selective process which separates the hyperplastic nodules from the neoplastic ones. The possibility cannot be ruled out that true autonomous neoplastic cells were present in some of the hyperplastic nodules in the mice that were killed soon after CCl_4 administration ceased but that we failed to select the right nodules for transplantation. However, in the mouse killed 11 weeks after CCl_4 treatments ended, the one large nodule present was distinct from the rest of the liver, which was almost normal histologically, yet it failed to grow in new hosts. This would seem to favor the concept that the primary hepatoma undergoes a progressive development or maturation which increases its capacity to grow in new hosts.

In our experience, which is usual with all hepatomas (*6*), there was a long latent period before the first generation of successfully transplanted hepatomas grew to a palpable size. Those transplanted subcutaneously from primary hepatoma #1 became palpable in 2 out of 6 recipients in 3 months, in a third mouse a month later, in 2 more mice after another month, but failed to take in a 6th mouse, through 12 months. The reason for these differences is not known. The cells of the primary tumor were not all exactly alike; there was a mosaic in which clusters of cells differed from surrounding ones in cell and nuclear size or glycogen and fat content. As Andervont and Dunn (*6*) pointed out, there may be a similar mosaic of growth potential which could be responsible for the varying results obtained when a series of transfers is made from the same tumor. On the other hand, the position of the implant in the new host, which was not precisely controlled in our mice, may be an important factor because during the latent period the implanted mass must become

revascularized and reorganized with respect to a new stroma. Andervont and Dunn (*6*) stated that an increase in growth rate has been observed during serial transplantation of all hepatomas and other tumors as well. In its first transplant generation, Hepatoma BC3 became palpable after 3 months, and was transplanted 1 month later. Now, after 14 subsequent intrasplenic passages, it has a latent period of about one month, that is, macroscopically visible nodules can be found in the spleen a month after implantation, and it reaches transplantable size 3 to 4 weeks later. Therefore, in this tumor the "increased growth rate" represents, largely, a decreased latent period. The only morphological changes that have occurred during serial passages have been a decrease in number of binucleate cells and a slight decrease in nuclear and cell size. These changes are illustrations of the progression in transplantable tumors that was reviewed by Foulds (*22*).

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PLATE 61

FIGURE 1.—Normal liver cells of an adult mouse. *Note* cell and nuclear size as compared with those in figures 2 and 3. Hematoxylin and eosin. × 750

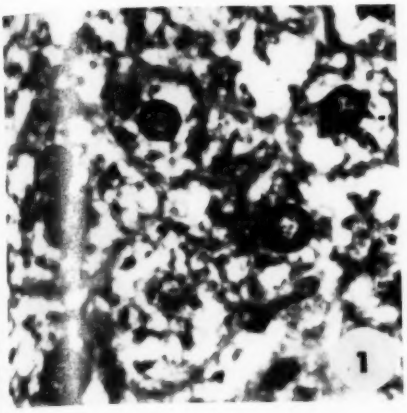
FIGURE 2.—CCl_4-induced primary hepatoma #1. Hematoxylin and eosin. × 750

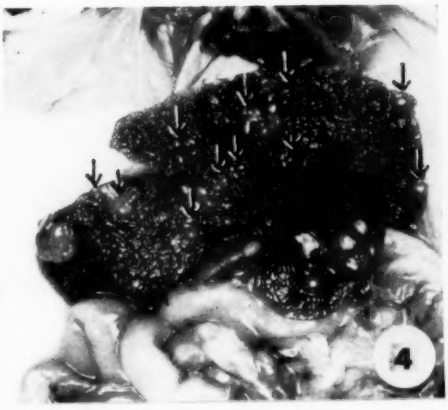
FIGURE 3.—Fourteenth intrasplenic transplant of the hepatoma in figure 2. Empty vacuoles represent sites of dissolved fat droplets. Hematoxylin and eosin. × 750

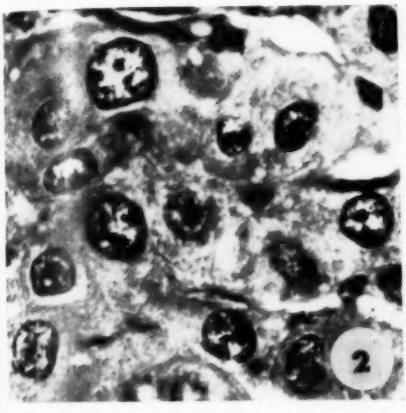
FIGURE 4.—Cirrhotic liver of a mouse 2 days after the 78th dose of CCl_4. *Arrows* indicate hyperplastic nodules. One hepatoma is on the caudal lobe of the liver.

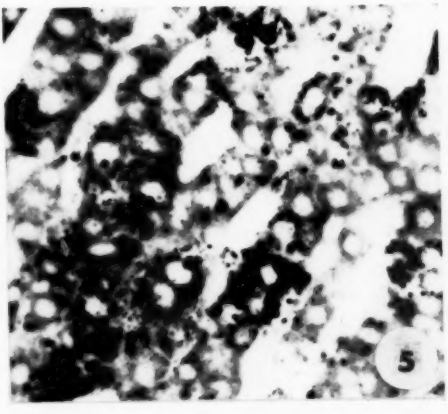
FIGURE 5.—Sudanophilic lipides (black droplets) in the fourteenth generation intrasplenic transplant of Hepatoma BC3. *Note* cords of cells and sinusoid-like blood spaces between them. Sudan black. × 500

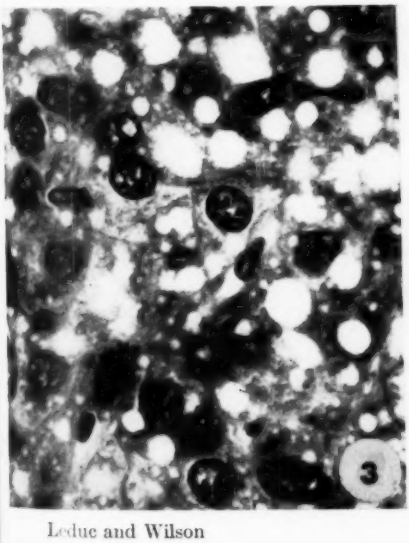
FIGURE 6.—Filamentous mitochondria in the twelfth generation transplant of Hepatoma BC3. *Arrows* indicate bile canaliculi and their associated terminal bars. An unusually large binucleate cell is shown. Regand's hematoxylin. × 1,125











Leduc and Wilson

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A Comparative Study of the Shope Fibroma in Rabbits in Relation to Transmissibility by Mosquitoes [1,2]

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Summary

A study of the Shope fibroma in cottontail rabbits, suckling domestic rabbits, and adult domestic rabbits revealed that the transmissibility of the fibroma by mosquitoes can be correlated with the histologic development of the lesion. The developing fibroma of the suckling domestic rabbits or of the cottontail rabbits generally became infective for mosquitoes when it reached a stage of maturity characterized by closely packed fibroma cells rich in cytoplasm containing inclusion bodies larger than 2 μ. The fibromas of adult domestic rabbits, induced by inoculating suspensions of fibroma tissue, regressed before reaching this mature stage and rarely were infective for mosquitoes. Fibromas that were infective for mosquitoes could be induced in the adult domestic rabbit by altering host-virus relationships through X irradiation or carcinogen treatment of the host, or passage of the virus through a foreign host. Such lesions became infective when prolongation of their development permitted maturation to a morphologic stage comparable to that seen in the mosquito-transmissible fibromas of suckling domestic rabbits and cottontail rabbits.—**J. Nat. Cancer Inst. 22: 593–615, 1959.**

Several different forms of biting insects fed on naturally occurring or experimentally induced Shope fibromas in cottontail rabbits, *Sylvilagus floridanus*, are capable of transmitting the fibroma to domestic rabbits, *Oryctolagus cuniculus* (*1–4*). It has been shown recently that the Shope fibroma, induced in the 7- to 9-day-old suckling domestic rabbit, can also be transmitted by biting insects to adult domestic rabbits (*4*). However, fibromas induced in adult domestic rabbits generally cannot be transmitted by biting insects or by subcutaneous inoculation with the mouthparts of insects that have fed on the fibromas (*3*). This difference in transmissibility in respect to the age and species of the host is probably not due to differences in the viral content of the lesion, since virus titers of the nontransmissible fibromas of the adult domestic rabbit are as

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nigh as the virus titers of the transmissible fibromas of cottontail and suckling domestic rabbits (*3*). Kilham and Dalmat (*3*) suggested that the variation in transmissibility by insects might be due to differences in tumor maturation. Thus, as the tumor becomes more fully developed, the virus particles might be nearer the surface and more accessible to the mouthparts of the insect.

This study was designed to determine 1) whether the morphologic differences in fibromas are related to the infectivity of the lesions for insects, 2) the extent of such morphologic differences in lesions in cottontail and domestic rabbits and in suckling and adult rabbits of the domestic species, and 3) whether fibromas which become transmissible in the adult domestic rabbit, following alteration in the host or the virus, possess the same cellular characteristics as those of transmissible lesions in the cottontail and suckling domestic rabbits.

Materials and Methods

Virus.—The virus used was of the Boerlage strain, originally obtained from Dr. Richard E. Shope, of the Rockefeller Institute for Medical Research. Passages were made in domestic rabbits with a standard 10 percent beef infusion-broth suspension of tumor tissue which had been stored in broth at −20° C. The standard inoculum was a suspension of this preparation given by intradermal injection in 0.25 ml. amounts to adult rabbits and 0.10 ml. amounts to suckling rabbits. Dilutions as high as 10^{-6} of this stock suspension induced characteristic lesions in domestic rabbits.

Rabbits.—The domestic rabbits were the New Zealand White strain of the European rabbit, *O. cuniculus*. These were bred by the Animal Production Section, of the National Institutes of Health. The adult domestic rabbits were 8 to 9 weeks old and weighed between 1800 and 2000 gm. when inoculated. The suckling rabbits were of the same strain and were 7 to 9 days old when inoculated. The cottontail rabbits were subspecies of *S. floridanus*. They were trapped by Mr. Paul Downing, Biologist for the Missouri Conservation Commission, and Dr. Carleton M. Herman, Chief of the Wildlife Pathology Section, Patuxent Research Refuge, United States Fish and Wildlife Service.

Observations were made on 40 cottontail rabbits and on 340 adult and 21 suckling domestic rabbits. The study of developmental morphology was made on tumors induced by the standard intradermal inoculation described. As many as 6 inoculations were made simultaneously in a single rabbit. The individual tumors removed at different times post inoculation permitted comparison of tumors developing in a single rabbit as well as tumors developing in different rabbits at the same time interval. Histologic observations and insect infectivity tests were made on tumors induced by the standard inoculum in untreated animals and in irradiated or carcinogen-treated animals. Similar studies were carried out on a few

lesions induced either by the bite of infected insects or by the inoculation of suspensions of infected mouse brain tissue.

Entomologic techniques.—Although several insects are capable of transmitting fibromatosis, only mosquitoes (*Aedes aegypti*) were used as test insects in the present study. The mosquitoes were 2 to 5 days old, and had not fed previously on animals. All colonies were kept in an insectary maintained at 78° F. and 60 percent relative humidity. The test of a fibroma's transmissibility was carried out as follows: The mosquitoes were transferred from the insectary, in groups of 10, to glass cylinders, measuring 1½ by 3 inches, both ends of which were covered with cotton netting. The cylinders were then held against the fibroma until all mosquitoes had fed. Two cylinders were used in each attempt at transmission. In some experiments, the mosquitoes were not fully fed on the fibroma but were tranferred to a noninfected adult domestic rabbit to complete their engorgement. In general, they were permitted to feed completely on the tumor, and after 2 or more days they were either refed on the shaved backs of noninfected test rabbits or a suspension of their pooled mouthparts (fig. 5) was inoculated intradermally. Twenty mouthparts triturated in 1.0 ml. of beef infusion broth constituted a 10 percent suspension for the purpose of titration.

Histologic techniques.—All tissues were fixed in Zenker-formol and embedded in paraffin. Sections were colored by Mayer's hematoxylin and eosin, Masson's trichrome stain, Shorr's triple stain, Wilder's silver method for reticulum, and the McManus periodic acid-Schiff reaction. A few sections were colored with the Rhinehart-Abul-Haj method for acid mucopolysaccharides.

Results

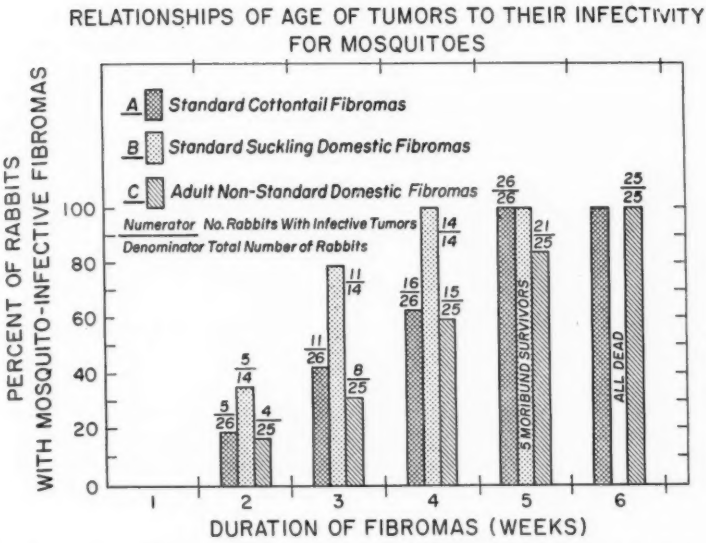
Infectivity of the Fibromas for Insects

Multiple lesions, induced by standard inoculums in 26 cottontail rabbits, 14 suckling domestic rabbits, and 200 adult domestic rabbits, were tested for their capacity to infect mosquitoes. This testing was done at irregular intervals, usually beginning 7 to 10 days after inoculation.

The data of table 1 confirm the earlier conclusion (*3*) that lesions induced in adult domestic rabbits by inoculation of the standard fibroma suspension are not infective for mosquitoes. None of the fibromas of the 200 adult domestic rabbits developed the capacity to infect the mosquito; in contrast, all the fibromas of the cottontail and suckling domestic rabbits did become infective after sufficient time elapsed. Columns 1 and 2 of text-figure 1 show the time at which the tested fibromas of the cottontail and suckling domestic rabbit became infective for mosquitoes. The average and median time for development of infectivity in the cottontail rabbits (natural host) was 24.6 and 25 days, respectively; in the suckling domestic rabbits, 17.2 and 16.5 days. The distribution was regular in both groups. However, insect-infectivity developed more rapidly in the suckling domestic rabbit than it did in the cottontail rabbit.

TABLE 1.—*Relationship of the cellular development of the fibroma to its infectivity for mosquitoes*

Fibromas transmissible by mosquitoes	Rabbit hosts	Time tested (*days post inoculation*)	Developmental status of the lesions
Fibromas induced by standard inoculation			
Yes	26 cottontails	10–35	Mature fibroma cells with marked marginal inflammation. Fibroma cell inclusions greater than 2 μ, epithelial inclusions present.
	14 domestic sucklings	10–25	Mature fibroma cells with minimal marginal inflammation. Fibroma cell inclusions greater than 2 μ, epithelial inclusions present.
No	26 cottontails	7–28	Proliferating immature fibroma cells with marked marginal inflammation. Fibroma cell inclusions less than 2 μ, epithelial inclusions absent.
	13 domestic sucklings	7–23	Proliferating immature fibroma cells with minimal marginal inflammation. Fibroma cell inclusions less than 2 μ, epithelial inclusions absent.
	1 domestic suckling	10	Transitional mixture of mature and immature fibroma cells without inflammation. Fibroma cell inclusions greater than 2 μ, epithelial inclusions present.
	200 domestic adults	7–14	Proliferating immature fibroma cells with marked marginal inflammation. Fibroma cell inclusions less than 2 μ, epithelial cell inclusions absent.
Fibromas induced after alterations of host or virus (table 3)			
Yes	25 domestic adults	10–40	Mature fibroma cells with marked marginal inflammation. Fibroma cell inclusions greater than 2 μ, epithelial inclusions present.
No	22 domestic adults	7–21	Proliferating immature fibroma cells with marked marginal inflammation. Fibroma cell inclusions less than 2 μ, epithelial inclusions absent.



TEXT-FIGURE 1.—The relationship of the age of fibromas to their infectivity for mosquitoes.

Pathogenesis of Fibromas Induced by Standard Inoculums

Gross Development

In the adult cottontail rabbit, the lesion begins as an inflammatory wheal, within the first 5 to 7 days following inoculation, and a definite mass of tissue forms at a much slower rate than the fibroma in the adult or suckling domestic rabbit. About 2 weeks after inoculation, if the tumor in the cottontail rabbit is sectioned, it is firm and fibrous and not viscid as it was earlier. The tumor reaches its maximum diameter (25 to 30 mm.) and its highest virus titer (infectivity at dilutions of 10^{-6} to 10^{-7}) about 3 weeks after inoculation (text-fig. 2). Usually the lesions of the cottontail rabbit subside sometime between the 3rd and 7th month, though they may persist for longer periods (fig. 1).

In the adult domestic rabbit, a swelling develops within 2 days after inoculation. When sectioned, the swollen tissue appears gelatinous. After 10 to 12 days, the fibroma is as large and has a viral titer as high as that attained by the cottontail fibroma in 3 weeks (fig. 3). The tumors persist as viscid masses until they regress during the 2nd or 3rd postinoculation week. Thus, the entire process is completed by the time the cottontail and suckling domestic rabbit fibromas are reaching their peak of development.

During the first 10 days the fibroma of the suckling domestic rabbit resembles the adult domestic rabbit lesion in consistency and virus titer. The fibroma, however, does not regress but continues to grow and assumes

the more firm consistency of the cottontail lesion (fig. 2). The inoculated suckling domestic rabbit usually dies within 3 to 4 weeks without evidence of a decrease in the size of the lesion, though varying degrees of ulceration are always found. Although the fibromas of the suckling domestic rabbits were initiated with less than half the adult rabbit dose, they exceeded the size of fibromas in the older rabbits, often reaching 50 mm. in diameter. The largest fibromas in the entire group occurred in a suckling domestic rabbit (*see* "Regression" under "Connective Tissue Response") which developed secondary fibromatous growths in the internal organs.

Connective Tissue Response

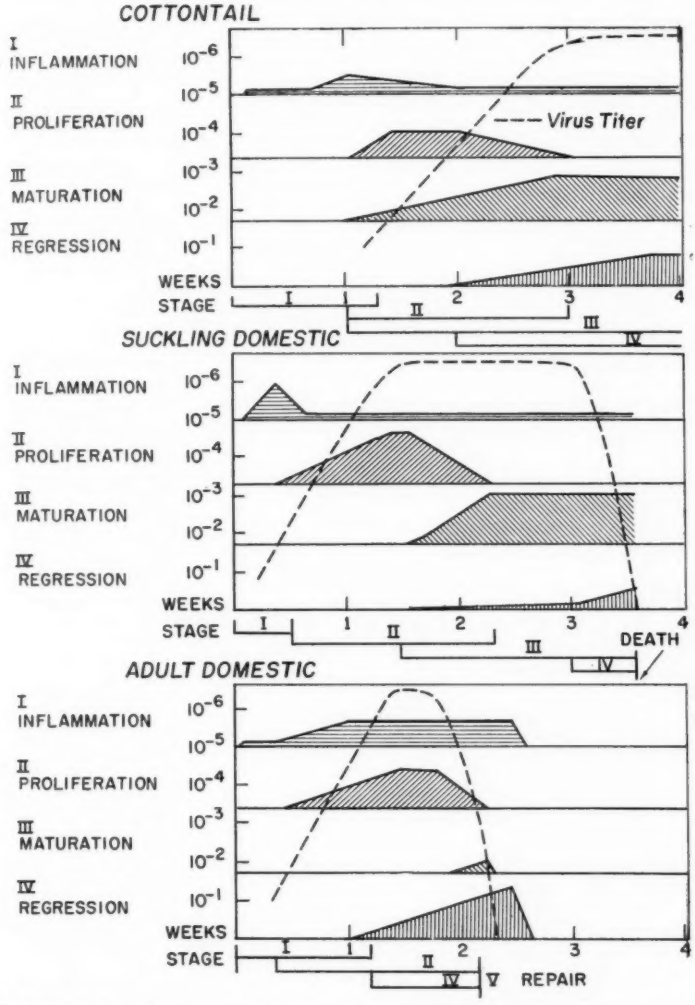
Four major changes in the cutaneous connective tissue were found to form a consistent sequential response to intracutaneous inoculation of the standard fibroma preparation. A detailed account of the histologic development is not required, since this has already been described by other investigators (*5–7*). However, the following paragraphs emphasize the criteria adopted in this study as arbitrary, but distinguishable dividing lines in separating the stages of fibroma evolution. Text-figure 2 records graphically the relative size of the lesions and the intensity of the 4 tissue responses to be described. The sequence of changes is similar in all 3 types of rabbit, though significant differences in the rate and intensity of the 4 cellular responses distinguish the lesions of the cottontail, the suckling domestic rabbit, and the adult domestic rabbit.

1) *The acute inflammatory stage.*—The earliest reaction consists of a local exudation of tissue fluid and inflammatory cells into the corium. There is little cellular necrosis, but varying degrees of polymorphonuclear leukocyte infiltration and hyperemia are present. The tissue reaction is the usual inflammatory response to nonspecific injury. Fibroma cells cannot be recognized (figs. 6 and 7).

The acute inflammatory stage has an earlier onset and is most intense in the suckling rabbit. However, in this young host all inflammatory reaction rapidly subsides as fibroma cell proliferation begins, so that significant inflammatory changes are rarely found after the first few days. In both the cottontail and adult domestic rabbit the acute inflammatory reaction merges with a granulomatous reaction that persists throughout the fibroma's existence.

2) *The proliferative stage.*—Small foci of proliferating cells, apparently derived from undifferentiated mesenchyme, arise within the area of inflammation. In the early proliferative phase the cells are focally distributed around small blood vessels and hair follicles, but they soon make up almost the entire cell population of the affected area. The proliferating cells are stellate with delicate strandlike cytoplasmic processes and elongated nuclei. The cytoplasm of the proliferating fibroma cells is never abundant at this stage, though some cells contain foamy cytoplasmic vacuoles. The vacuoles appear to contribute to the formation of an extensive intercellular matrix of mucoid material. This probably accounts for the grossly viscid consistency of the lesion. Neither the vacuoles nor

the intercellular ground substance was colored in microscopic sections treated by the histologic techniques listed for this study. Argentophilic fibrils, probably representing residual reticulum dispersed by the expanding lesion, were found scattered within the mucoid matrix. No satisfac-



TEXT-FIGURE 2.—Diagrammatic pattern of connective-tissue response after intradermal injections of standard fibroma suspensions of 10^{-6} infectivity titer.

tory evidence that these fibrils contributed to the formation of new collagen could be found. The lesion at this stage may extend down through the panniculus carnosus to involve the entire thickness of the subcutaneous tissue. Tumorlike masses of considerable proportion are invariably

formed. It is in this stage that the fibroma most closely simulates the local lesion of infectious myxomatosis (figs. 8 and 9).

The proliferative stage is less prominent and develops later in the cottontail than in either young or old domestic rabbits. However, it persists longer in the cottontail, usually throughout the 2nd and 3rd postinoculation weeks, while the similar developmental phase of the suckling domestic rabbit lesion has given way to a stage of maturation, and the adult domestic rabbit lesion is rapidly regressing.

3) *The maturation stage.*—As growth continues, a more compact lesion is formed as the result of an increase in the number of fibroma cells and a marked increase in their cytoplasmic volume. Many cells show mitotic figures, and bizarre forms are present. The cells appear more fibroblastic in this stage because of an abundant acidophilic cytoplasm, fusiform shape, and elongated nuclei oriented with the long axis of the cell body. In the larger lesions the panniculus carnosus is destroyed and the underlying skeletal muscle may be involved. The increase in cell size and number obliterates the mucoid matrix characteristic of the proliferative stage. At this phase of development the lesion most closely resembles true neoplasia (figs. 10 through 15).

This maturation of the fibroma cells was seldom found in lesions of the adult domestic rabbit. It is only in the last days preceding complete regression of the adult domestic lesion that a few of the fibroma cells form appreciable amounts of cytoplasm. Although maturation begins later in the suckling domestic lesion than in the cottontail lesion, it develops in the former at a more rapid rate so that all the fibroma cells of the suckling domestic rabbit show this change fully a week before the cells of the cottontail lesion reach an equivalent phase of development.

4) *Regression.*—The lesion regresses from central expanding foci of fibroma cells undergoing necrosis. Independent of this central necrosis and usually before its appearance, a distinct granulomatous boundary forms at the periphery of the cellular mass. The margin of the fibroma, which showed evidence of varying degrees of infiltration with mononuclear cells throughout its development, now becomes populated with macrophages which assume the plump cytoplasm and processes of epithelioid cells. The phagocytic properties of these marginal cells are indicated by the presence of mineral inclusions and particulate debris within the cytoplasm, and some phagocytes fuse to form foreign-body giant cells. The origin of the phagocytes is difficult to determine, but transitional changes in the fibroma cells at the periphery suggest that the phagocytes may be derived from fibroma cells (figs. 16 and 17).

The marginal granulomatous response in cottontail and adult domestic rabbits marks the limits of the fibromas in all stages of development. In the suckling rabbit, in contrast, the relative lack of such a marginal barrier and the ability of the altered mesenchymal cells to penetrate the panniculus carnosus were characteristic. Mature lesions in 2 of the suckling rabbits that died 3 and 4 weeks after inoculation contained characteristic fibroma cell masses within the lumens of large venules at

the margins of the primary lesion (fig. 18), and there was deep penetration of the subcutaneous tissue also. In 1 of these 2 animals, metastatic foci of fibroma cells were found in an axillary lymph node, the lungs, the liver, and the kidneys (fig. 19). The fibroma cells in these distant lesions had morphologic characteristics and cytoplasmic inclusions similar to the cells in the inoculated cutaneous site.

5) *Repair.*—The necrotic mass of fibromatous tissue is dislodged from the ulcerated surface or resorbed. An ulcer base may be left behind which, in some cases, contains a few fibroma cells. As a rule, however, the residual tissue is composed only of the granulomatous boundary. Following the regression of the lesion, a secondary epidermal cyst occasionally forms. This may grossly resemble the original lesion and may persist for an indefinite period. These cysts probably arise from trapped fragments of the elongated rete pegs or from marginal epithelium which has grown down into the ulcer base. The cysts do not show characteristics of the fibroma either in their epithelial lining or surrounding connective tissue.

Intracytoplasmic Inclusion Bodies of the Fibroma Cell

Hyde (*8*) and Ahlström (*6*) first reported discrete particles within the cytoplasm of both the fibroma cells and the cells of the overlying epithelium. The histochemical nature of these particles has been thoroughly studied by Fisher (*9*) who demonstrated the intense fuchsinophilia of the fibroma-cell inclusion body when treated with periodic acid-leucofuchsin—a response not shared by the epithelial-cell inclusion body.

With the use of the periodic acid-Schiff technique, the development of the inclusion body in the fibroma cell was correlated with the stage of fibroma-cell maturation. Where comparable stages of the lesion can be studied, there are no differences in the character or quantity of the inclusion bodies of fibroma cells in the 3 groups of animals. No inclusions are evident in the stage of *acute inflammation*. When the proliferating stellate cells first appear, their cytoplasm contains sparsely scattered periodic acid-Schiff-colored granules which measure less than one μ in diameter. Older lesions contain a larger number of granules, which seem to increase so rapidly that the cytoplasm of most of the affected stellate cells is almost completely filled with finely granular inclusion bodies in the late stages of proliferation. During maturation the increase in particles keeps pace with the increase in the cytoplasmic volume of the fibroma cell. Furthermore, the granules coalesce to form large regular masses within individual cells (fig. 13). The largest of these cytoplasmic masses measure up to 18 μ in diameter and are surrounded by clear halos. In the stage of maturation, it is evident that the older lesions contain the largest inclusions. There is an increase in both the number and size of the particles in older lesions. The proportion of the mass containing inclusion bodies, rather than the size of individual bodies, may be of greater significance, however. With the technique of Chalkley (*10*), an increase

in the proportion of the tumor volume occupied by inclusion bodies can be shown to correlate with the advancing stages of the lesion. Table 2 shows that in lesions sampled from all 3 groups of animals, this quantitative increase in cytoplasmic inclusion bodies occurs with the maturation of the lesion. The most rapid formation of cytoplasmic particles is manifest during the transition from the stage of proliferation to the stage of maturation, when a notable increase in cytoplasm of the fibroma cells and loss of intercellular matrix are taking place. However, observations of PAS-stained sections reveal that the accumulation of inclusion bodies continues during the later stages of maturation and regression. This is noteworthy because no increase in the virus titers of the lesions is detected after the proliferative stage is reached. The development of a granulomatous reaction at the periphery apparently does not appear to affect the increasing population of inclusion particles in most of the fibroma cells. However, in those cases in which the granulomatous

TABLE 2.—*Fraction of fibroma mass composed of cytoplasmic inclusion bodies in lesions induced by the standard inoculation of tumor suspension*

	Proliferative stage				Mature stage				
Tumor age (*days*):	7	11	12	13	17	21	28	36	203
Adult cottontail		.04		.05		.08		.30	.64
Suckling domestic	<.01		.04		.11		.24		
Adult domestic	.03	.02	.03	<.01	(*)	(*)	(*)	(*)	(*)

*Adult domestic rabbit fibromas did not reach this stage.

reaction continued, a further concentration of PAS-colored particles is found in the phagocytic cells at the margin of the lesion. At the base, the cytoplasm of both fibroma cells and phagocytic macrophages is obliterated by masses of intracytoplasmic inclusion material. At the surface of the lesion, the layers of affected mesenchymal cells just beneath the epithelium show a bright-red band of PAS-positive material easily distinguishable at 30 magnifications. This indicates that the development of the inclusion bodies is parallel with the development of the fibroma cell and that there is a progressive increase in cytoplasmic particles as the fibroma cell matures. The pattern of fibroma-cell inclusion bodies is the same in the lesions of all 3 groups of rabbits in which comparable stages are available for study. However, since the lesions of the adult domestic rabbit fail to reach the stage of maturation attained in the other groups, these lesions also fail to develop the high proportion of cytoplasmic inclusions that are found in mature lesions of the suckling domestic and the cottontail rabbit.

Variations in the Overlying Epithelium

During early development thinning and elongation of the rete pegs with cellular distortion and increased mitotic activity are found to be common to the epithelium overlying lesions from all types of rabbits. Atrophy

of the adnexal structures, probably the result of compression, also follows the rapid swelling of the corium at this stage (fig. 10).

Cytoplasmic inclusion bodies within epithelial cells first appear during the stage of maturation. The inclusion bodies of the epithelial cells differ from those of the fibroma cells in their poor affinity for the PAS stain and in their larger size. When mature, they consist of granules of material which stain orange with the Shorr stain and red with the Masson trichrome stain. The inclusions are found within vacuoles which occupy much of the cytoplasm. In older lesions, a larger number of epithelial cells contains inclusion bodies, a feature particularly evident in the persistent fibromas of cottontail rabbits (figs. 12, 13). Lesions in the cottontail rabbit contain epithelial inclusion bodies 3 weeks after inoculation; the skin over lesions of suckling domestic rabbits contains inclusion bodies 2 weeks after inoculation; while no epithelial inclusion bodies are found in the epidermis over the fibromas of the adult domestic rabbit. In one cottontail, in which the lesion persisted for 203 days, a remarkable hyperplasia of the inclusion-laden dyskeratotic epithelium was found. This alteration extended for several centimeters from the site of inoculation and was sufficiently pronounced to suggest a preneoplastic transformation (fig. 12).

Although epithelial inclusions are not seen ordinarily in the lesions of the adult domestic rabbit, a unique reaction in the epithelial tissue above the lesion is noted at the peak of fibroma development. Beginning 2 weeks after inoculation, the surface epithelium shows marked intra-epithelial vesication, which is a change not found in fibromas of the cottontail or suckling rabbits (fig. 6). The vesicular lesion begins with vacuolation and edema in the cells of the malpighian layer. This change leads to the formation of fluid-filled vesicles which in turn become invaded by leukocytes—a process resulting in extensive ulceration of the surface. The intra-epithelial vesication and subsequent ulceration do not seem to be related to the size of the lesion. The epithelial vesication is the only morphologic response peculiar to the lesion of the adult domestic rabbit.

Correlation of the Structure of the Fibroma With Its Capacity To Infect the Mosquito

The capacity of the fibroma to infect the mosquito was found to be directly related to the developmental stage of the lesion (*cf.* table 1). The infective fibromas of both the adult cottontail rabbit and of the suckling domestic rabbit had, in common, the cellular qualities described as maturation, namely, the replacement of the mucoid stroma and proliferating stellate cells by closely packed fibroma cells rich in cytoplasm. The granulomatous reaction at the margin of the tumor was found in infectious tumors from cottontail rabbits, but it was absent or minimal in tumors from suckling domestic rabbits. The fibroma cells of infectious tumors of both groups had abundant cytoplasmic inclusion particles—large spherical bodies measuring more than 2 μ in diameter. The quantity of inclusion material coincided with the degree of fibroma-cell maturation. Inclusion

bodies were present in the epithelium overlying the fibromas of both cottontail rabbits and suckling domestic rabbits.

The noninfectious tumors from all 3 groups of rabbits lacked the histologic features described under the section on maturation. Such fibromas contained only a few intracytoplasmic inclusion bodies, always less than 2 μ in diameter. The overlying epithelium of the noninfectious tumors in all groups of rabbits had no inclusion bodies, though the epithelium overlying the tumors of adult domestic rabbits showed intra-epithelial vesication and ulceration. The noninfectious lesions in cottontail rabbits and suckling domestic rabbits revealed either abundant mucoid stroma with young proliferating stellate cells or, in the older lesions, a similar proliferative response with an extensive marginal granulomatous reaction and central necrosis. A single, 10-day-old noninfectious tumor from a suckling domestic rabbit showed a transition between the stage of proliferation and the stage of maturation. However, other tumors with identical external appearance on the same rabbit were infectious. Poor feeding by the mosquito or some technical error may account for this exception. The consistently noninfectious lesions produced by the standard inoculum in adult domestic rabbits contained proliferating stellate cells with scant cytoplasms, scattered inclusions less than 2 μ in diameter, a prominent intercellular mucoid matrix, and, invariably, a marked granulomatous reaction at the periphery of the tumor. These lesions did not progress beyond the stage of proliferation, though some parts of them showed evidence of regression.

A further correlation between insect infectivity and fibroma maturation can be made by comparing text-figures 1 and 2. This comparison indicates that the average time necessary for lesions of suckling rabbits or adult cottontail rabbits to develop the capacity to infect insects is very near the time required for such lesions to reach the final stage of maturation. The delay in the maturation of the lesion in the cottontail rabbit compared with the lesion maturation of the suckling domestic rabbit is reflected in a delay in the average onset of insect infectivity in the cottontail fibroma as compared with that in the suckling-rabbit fibroma.

Correlation of Morphology and Insect Infectivity of Domestic Rabbit Fibromas Induced by Host-Virus Alterations

The duration of fibromas in the adult domestic rabbit can be prolonged by inducing the lesion through the bite of infected insects (*4*), the inoculation of infected brain tissue of suckling mice (*11*), or by pre-inoculation exposure of the rabbit to X irradiation (*12*) or carcinogenic agents (*13*). Dalmat (*14*) found that these more persistent lesions of the adult domestic rabbit, unlike the lesion induced by fibroma suspensions in untreated animals, also were infective for mosquitoes.

Mosquito-infective lesions induced by such techniques (fig. 4) were included in this study to determine whether their histologic structure was comparable to that of the infective fibromas of the cottontail and suckling domestic rabbits. In our experience, 25 of 47 adult domestic

rabbits treated by such means have developed fibromas capable of infecting mosquitoes (text-fig. 1 and table 1). The method of treating rabbits that developed infective lesions is listed in table 3, along with the time when the fibromas became infective. Adjacent lesions on the same rabbit or lesions showing similar gross development from identically treated rabbits were removed for histologic study at the time when infectivity tests were carried out. Infective tumors or their adjacent counterparts were composed of closely packed fibroma cells, filled with large cytoplasmic inclusion bodies, identical to the mature form of the lesion in infectious cottontail and suckling domestic rabbits. Epithelial vesication and ulceration were usually present, but over some fibromas the epithelium contained cytoplasmic inclusion bodies (table 1; figs. 10 and 16). The noninfectious lesions from 22 of the treated adult domestic rabbits all showed phases of proliferation and regression like the noninfectious lesions induced in adult domestic rabbits by the standard fibroma inoculum, and did not show evidence of mature fibroma-cell formation.

TABLE 3.—*Methods of inducing the infective tumors of 25 adult domestic rabbits with the onset of their infectivity for mosquitoes*

Host-virus alterations (*method of induction*)	Number of rabbits	Interval from inoculation to mosquito infectivity (*days*)
Irradiated rabbits	9	10, 10, 15, 26, 27, 27, 29, 33, 36
Methylcholanthrene-treated rabbits	7	20, 20, 30, 32, 32, 36, 40
1,2,5,6-dibenzanthracene*-treated rabbits	1	26
Bite of infected mosquitoes	2	22, 27
Heads of infected mosquitoes	2	25, 39
Heads of infected reduviid bugs	2	12, 19
Infected mouse brain suspension	1	15
Suspension of fibroma induced by mosquito bite	1	14

**Chemical Abstracts* nomenclature: Dibenz[*a,h*]anthracene.

Discussion

It is apparent that little morphologic difference can be detected in fibromas developing in cottontail rabbits, adult domestic rabbits, and suckling domestic rabbits when similar stages in development are studied. An exception is the intra-epithelial vesication which occurs exclusively in the overlying epithelium of the adult domestic-rabbit lesion. However, significant differences were noted in the rate and duration of the various phases of development in the fibromas of the 3 rabbit groups. The most striking contrast is apparent between the lesions of the suckling rabbit and those of the adult domestic rabbit, induced by equivalent inoculums. The suckling rabbit rapidly develops a lethal tumor poorly demarcated by inflammatory reaction, and composed of mature, inclusion-filled fibroma cells which possess invasive properties and high mitotic activity. In contrast, the lesion of the adult domestic rabbit begins at about the

same rate of development, but never reaches maturity, never abounds in inclusion material, and, for the duration of its short existence, is restricted by a vigorous inflammatory reaction. Between these 2 extremes lies the fibroma of the adult cottontail rabbit. It develops characteristics of maturation and persistence like those of the fibroma of the suckling rabbit, although at a somewhat slower rate, but is also characterized by the vigorous marginal inflammatory reaction and inevitable regression seen in the fibroma of the adult domestic rabbit. These differences in fibroma development in the 3 groups of rabbits probably reflect differences in both the host and the virus. In the adult domestic rabbit, for example, altering either of these factors causes the fibroma to develop and mature in a manner much like the cottontail fibroma (text-fig. 1).

In an earlier publication (*3*) Kilham and Dalmat concluded that differences in the degree of cellular maturation might be responsible for differences in insect infectivity. The present study supports this opinion. It is clear that mosquito-infective fibromas from cottontail, suckling, or adult domestic rabbits show an advanced state of structural development characterized by closely packed, cytoplasm-rich fibroma cells possessing a high content of large, PAS-positive inclusion bodies. These characteristics are not found in fibromas of cottontail, suckling, or adult domestic rabbits that have not become infective for mosquitoes. It has been shown that lesions induced in adult domestic rabbits cannot be transmitted by insects. Our histologic analysis offers an explanation, since we observed that fibromas induced by the usual method (inoculating a suspension of fibroma tissue) generally regress early before attaining the degree of maturation essential for the mosquito to transmit them. When the duration of fibromas in the adult domestic rabbit is prolonged by altering either the virus or the resistance of the host, the lesions frequently show mature cellular development, seen in transmissible suckling and cottontail lesions, and are infective for the mosquito.

It can be shown, by inoculating domestic rabbits with serially diluted suspensions of fibromas, that the virus content of fibromas from all 3 groups of rabbits, including the consistently nontransmissible lesions of the adult domestic rabbit, reaches the same high titer (10^{-6} to 10^{-7}) long before the lesions are infective for insects (*3*, *15*) and presumably before complete cellular maturation and inclusion development are attained (text-fig. 2). This indicates that the insect infectivity of the fibroma follows the pattern of cellular maturation and inclusion development rather than the virus content of the lesion. The latter appears to be the factor involved in the transmissibility of other virus-induced proliferative lesions of the rabbit, such as myxomatosis (*16*, *17*) and papillomatosis (*18*, *19*). Either some quality of the immature fibroma, such as the excessive intercellular matrix, is active in preventing the retention of virus by the mosquito or some quality of the mature fibroma enables the mosquito to retain the virus. The relation of the large inclusion particles in the mature fibroma cell to the virus has not been established, though our studies indicate that they are not directly related to the viral content of

the lesion. Fisher (*9*) also determined that these bodies do not have the histochemical properties of viruses. It may be that such particles, although not strictly viral in nature, are essential to insect infectivity by acting as a scaffold which permits the virus to be retained by the insect.

Although large cytoplasmic inclusion bodies within the epithelial cells are found only over insect-infective fibromas, their significance in the infectious process has not been ascertained. The presence of these epithelial-cell inclusions, however, indicates that the fibroma beneath probably is infective for mosquitoes.

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PLATE 62

FIGURE 1.—Thirty-nine-day-old fibroma in an eastern cottontail rabbit induced by intradermal inoculations of a standard fibroma suspension. These tumors were still in excellent condition and infective for mosquitoes when removed at 203 days. × 0.75

FIGURE 2.—Seventeen-day-old fibromas in a suckling domestic rabbit induced by intradermal inoculations of a standard fibroma suspension. × 1

FIGURE 3.—Eight-day-old fibromas in an adult domestic rabbit induced by intradermal inoculations of a standard fibroma suspension. × 1

FIGURE 4.—Thirty-four-day-old fibromas in an X-irradiated adult domestic rabbit induced by intradermal inoculations of a standard fibroma suspension. × 1

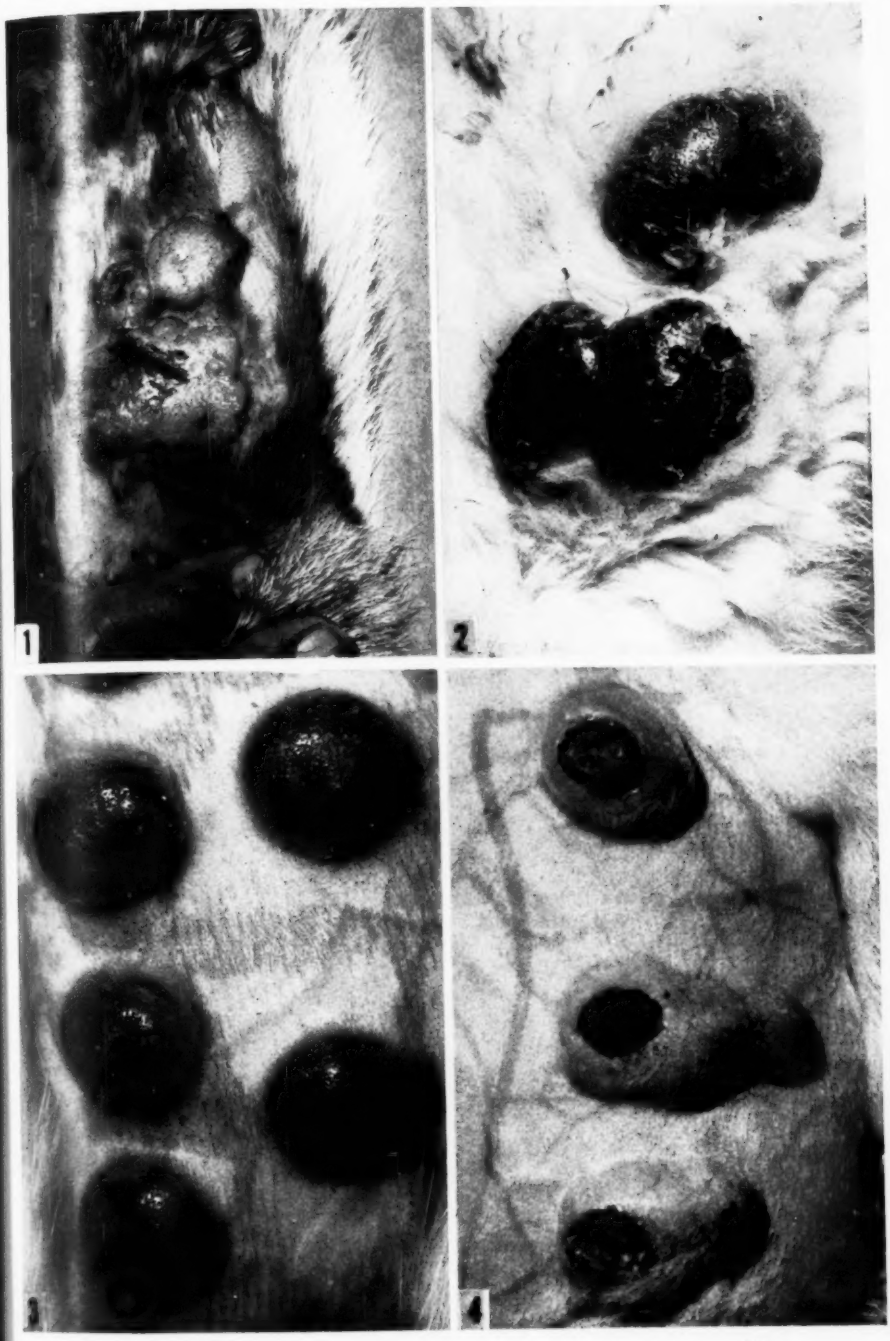

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PLATE 63

FIGURE 5.—Head and mouthparts of the mosquito, *Aedes aegypti*. Same range of magnification as figures 6, 8, 10, 14, and 16. × 35

FIGURE 6.—Adult cottontail rabbit, 8 days post inoculation with standard fibroma dose. Primary inflammatory reaction in a lesion that was noninfective for mosquitoes. Hematoxylin and eosin. × 37

FIGURE 7.—Higher magnification of figure 6. Hematoxylin and eosin. × 290

FIGURE 8.—Suckling domestic rabbit, 7 days post inoculation with standard fibroma dose. Early proliferative stage of a lesion that was noninfective for mosquitoes. Hematoxylin and eosin. × 40

FIGURE 9.—Higher magnification of figure 8. The field shows 2 fibroma cells with foamy vacuolated cytoplasm. Most of the fibroma cells have little cytoplasm and are widely separated by the mucoid matrix. × 290

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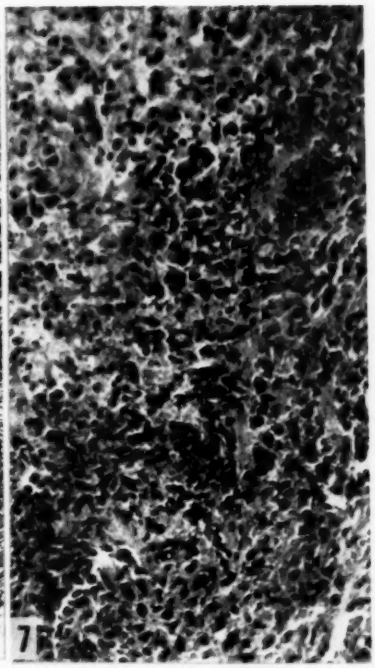
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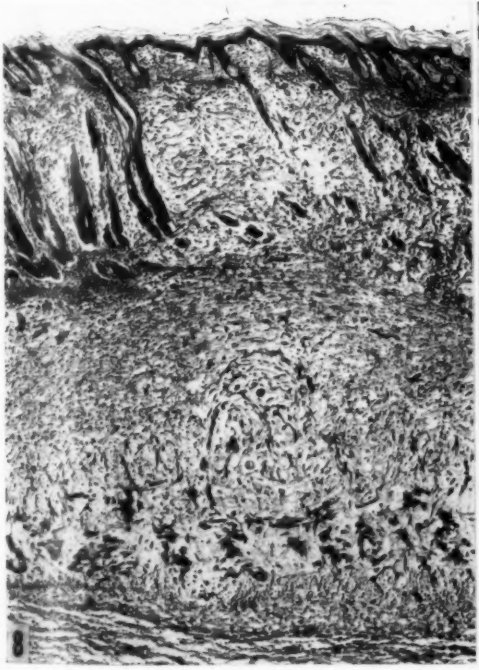
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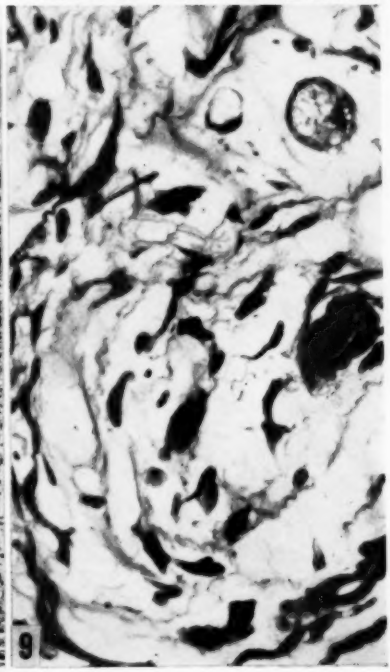
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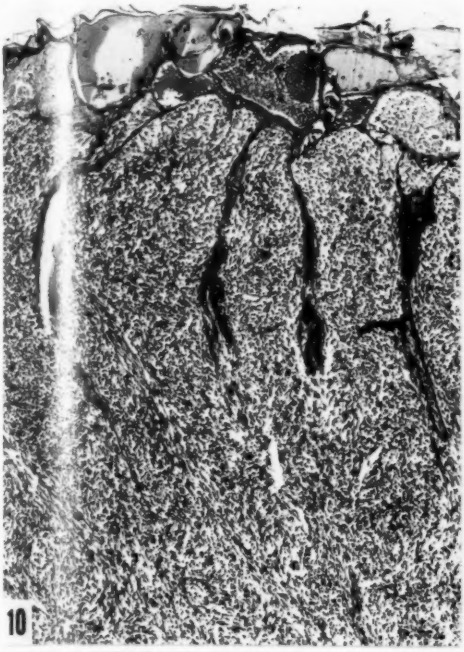
PLATE 64

FIGURE 10.—Early stage of maturation showing the transition of proliferating cells to mature fibroma cells in a mosquito-infective 16-day-old lesion at site of infected mouse-brain inoculation in an adult domestic rabbit. *Note* intra-epithelial vesiculation characteristic of adult domestic-rabbit fibromas. Hematoxylin and eosin. × 40

FIGURE 11.—Higher magnification of figure 10. The cells have increased their cytoplasmic volume and contain scattered cytoplasmic inclusions. × 290

FIGURE 12.—Late stage of maturation in a mosquito-infective 203-day-old lesion at site of standard fibroma inoculation in an adult cottontail rabbit. Both the fibroma cells and the epithelium contain many large cytoplasmic inclusions. The epithelium shows exceptional hyperplasia. Masson's trichrome stain. × 70

FIGURE 13.—Higher magnification of figure 12. Large fibroma-cell inclusions are illustrated at *bottom* of photograph. Epithelial inclusions are at *top*. × 290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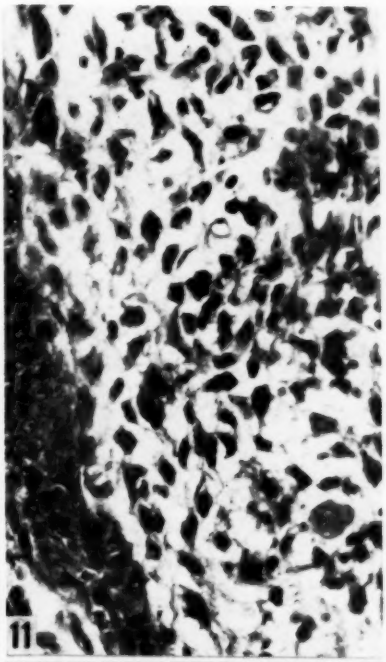
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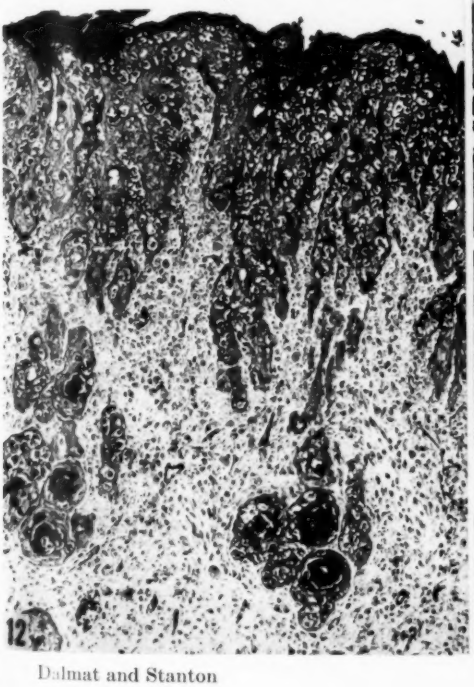
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Plate 65

Figure 14.—Late stage of maturation and regression in a mosquito-infective 21-day-old lesion at site of standard fibroma inoculation in a suckling domestic rabbit. The surface has ulcerated in area illustrated. Hematoxylin and eosin. × 35

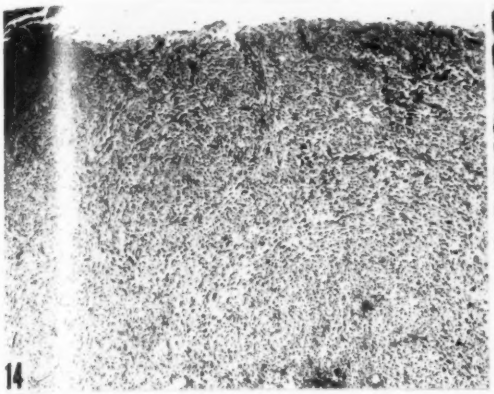
Figure 15.—Higher magnification of figure 14. *Note* character of the fibroma cells and their cytoplasmic inclusions which at this stage of development lie between the fibroma cells illustrated in figures 11 and 13. × 290

Figure 16.—Stage of regression in a mosquito-infective 39-day-old lesion at site of inoculation of infected mosquito heads in an adult domestic rabbit. Central area is ulcerating. Periphery is composed of fibroma cells similar to those in figure 15 bounded by a granulomatous margin. Hematoxylin and eosin. × 40

Figure 17.—Higher magnification of granulomatous margin of lesion illustrated in figure 16. The cells are filled with inclusion material and other phagocytized particles. × 290

Figure 18.—Growth of fibroma cells in a venule at base of a 17-day-old lesion composed of mature fibroma cells in a suckling domestic rabbit. Hematoxylin and eosin. × 130

Figure 19.—Metastatic growth of fibroma cells in the kidney of the same rabbit illustrated in figure 18. Hematoxylin and eosin. × 84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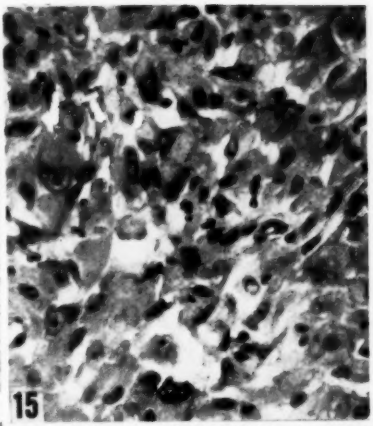
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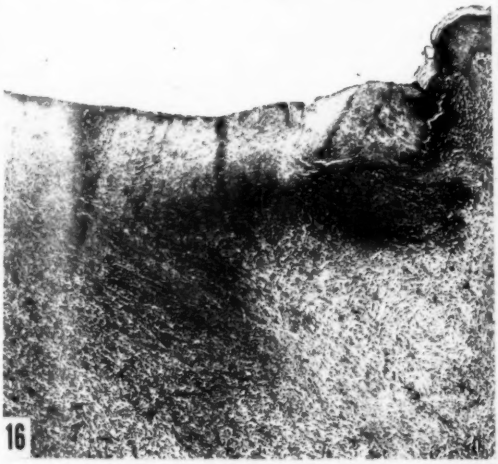
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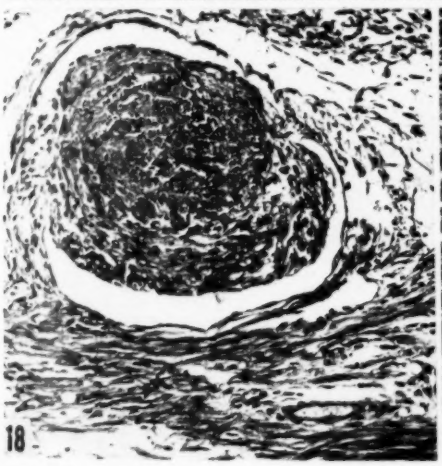
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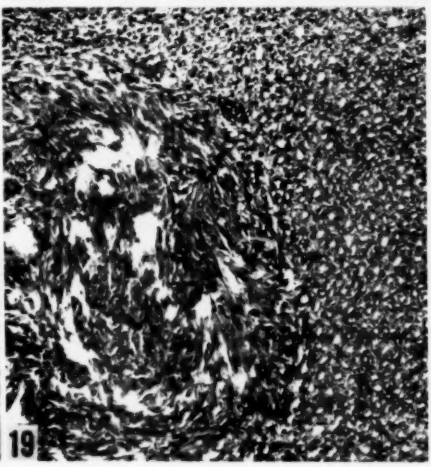
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A Study of the Frequency of Achlorhydria Among Japanese in Los Angeles[1]

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Summary

A survey of the frequency of achlorhydria was conducted among Japanese in Los Angeles, together with Caucasian and Mexican control subjects. One or more tubeless gastric analyses employing the azure-A exchange resin were performed on 717 Japanese subjects and 318 control subjects of Caucasian and Mexican origin. Of these 1,035 subjects, the data on 26 were excluded from consideration because of a history of previous gastric surgery, and on 46 because of diagnoses of gastric pathology. The Japanese are of especial interest with regard to achlorhydria because of their threefold increased risk to gastric cancer. The frequency of achlorhydria among *isei* was compared with that among *nisei*, and no significant difference could be demonstrated. Similarly, the frequency of achlorhydria among all the Japanese as compared to the Caucasian controls indicated there is no difference. However, the data presently available for the Mexican control group indicates that achlorhydria is more frequent in this group than in either the Japanese or Caucasians. Final interpretation of this finding should be reserved until additional data are secured. The reason for the difference in frequencies of achlorhydria in different study groups is discussed as is the reliability of this tubeless gastric analysis. It is felt by the authors that this simple test is quite reliable and that when several results in the same patient differ, this is not due so much to an inherent defect of the test as to the fact that the patient's acid secretory ability changes from time to time.—**J. Nat. Cancer Inst. 22: 617–631, 1959.**

It now has been well established that the Japanese people have a considerably higher risk to gastric cancer than do people of other racial groups. Nagayo found that 43.7 percent of all carcinomas in Tokyo were of gastric origin (*1*). In a more recent study of cancer morbidity in

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a defined geographic area of Japan, Segi *et al.* compared age-adjusted cancer incidence rates in Miyagi Prefecture, Japan, and 10 metropolitan areas in the United States. The relative frequency was found to be such that for every 100 gastric cancers among United States males, 339.3 would have occurred in Japanese males; and for every 100 United States females with gastric cancer, there would have occurred 292.5 cases among Japanese females (*2*). Steiner first demonstrated the higher risk to gastric cancer among Japanese in this country. In his autopsy series at the Los Angeles County General Hospital he found 21 gastric cancers. These constituted 36.2 percent of all malignant tumors and 8.8 percent of all necropsies among the Japanese (*3*). Smith emphasized the greater risk to gastric cancer among Japanese in this country, but presented evidence to the effect that the disease is significantly less frequent among Japanese in this country than it is among Japanese in Japan (*4*). In California there were 90 deaths from gastric cancer among Japanese men in the period 1949–51, whereas if the age-specific death rates from gastric cancer for all California males during this period had been experienced by Japanese men they would have been expected to have had only 33 deaths from this cause. For Japanese women 21 gastric-cancer deaths occurred, whereas only 7 would have been expected from the experience of all California women (*5*).

Von den Velden first reported the absence of free acid in the stomachs of patients with gastric cancer in 1879 (*6*). Since that time, a considerable body of literature has been published in support of the contention that there is a relationship between achlorhydria and gastric cancer (*7–19*). The frequency of achlorhydria in large population groups is between 10 and 22.3 percent, depending on the age distribution of the population groups and the type of gastric secretory stimulant used (*17–24*). In patients with gastric cancer the frequency varies between 50 and 90 percent, depending again on the selection of cases and the type of stimulant used (*8, 14, 19, 25, 26*).

The present study was conducted to determine whether or not there is a higher frequency of achlorhydria within a Japanese population to correspond with the approximately threefold increased risk to gastric cancer. The survey was conducted in Los Angeles, where there is a large, stable Japanese community. At the time of the 1950 census there were 25,502 Japanese in the city of Los Angeles—13,436 males and 12,066 females. In addition, there were over 11,000 Japanese in Los Angeles County, outside the city proper.

Present Study

Of 717 Japanese subjects on whom 1 or more gastric analyses were performed, 122 were inpatients at the Japanese Hospital in Los Angeles and the remainder volunteered for the test after reading newspaper accounts of the study or at the suggestion of the private Japanese physicians who cooperated in the study. All reports of study findings were made only to the private or attending physicians of the study subjects. The

control groups were made up of Caucasian subjects, primarily of Western European stock, and people of Mexican origin, who represent various admixtures of Caucasian and Mongoloid (American Indian) stocks. Negroes were excluded from this study. There were 318 control subjects: 263 were inpatients at the Los Angeles County General Hospital or non-Japanese admissions to the Japanese Hospital, 37 were outpatients at the Los Angeles County General Hospital, and the remainder were volunteers. The smaller number of controls, as compared to the number of Japanese studied up to the present, resulted from the time technicians had available. First consideration was given to the Japanese available for testing and the remaining time was devoted to controls. This inequity will be brought into balance as work progresses.

A subject qualified for the examination if he or she were 20 years of age or more and were of Caucasian or Mexican origin, or Japanese. Whether subjects were inpatients, outpatients, or volunteers, no attempt was made to select on the basis of pathology, when known, and no subject was denied the test providing he met the stated criteria. In the data presented here, however, patients have been excluded who had conditions which might compromise the accuracy of the test, such as uremia, liver failure, upper intestinal obstruction, or renal obstruction (*27*). The results on the 25 subjects who had previous gastric surgery (pyloroplasty, vagotomy, gastrectomy) have been excluded from the analysis because of the effect such surgery might have on the result of the test (*28*).

In addition, there were patients in each group who had, or eventually were found to have, some form of gastric pathology. There were 46 of these patients altogether—11 Japanese, 23 Caucasians, 12 Mexicans. Peptic ulcers predominated as the diagnosis among them, with a few instances of polyps and gastritis. One patient with pernicious anemia was achlorhydric, as would be anticipated. Of 3 with gastric cancer only 1 was achlorhydric. Inclusion or exclusion of the data from these cases, on the whole, made little difference in the analysis of results since diagnoses usually associated with normochlorhydric or hyperchlorhydric states predominated. At the present time, however, the data from Mexican subjects show a statistically different frequency of achlorhydria in exceeding that of each of the other groups. This was true when the cases with gastric pathology were included as well as when they were excluded, as they will be in all further considerations of the data presented here.

Materials and Methods

The presence or absence of free HCl was determined by the tubeless method, with the azure-A ion-exchange resin.[6] As far as qualitative determination of the presence of free HCl is concerned, the tubeless method has been shown to correlate quite satisfactorily with the standard intubation method of gastric analysis (*29–34*).

Procedure.—The tubeless gastric analysis is conducted as follows: Prior to the test, from midnight on, the subject is directed to fast and to

[6] This product is marketed under the trade name Diagnex Blue (Squibb).

abstain from cigarette smoking and gum chewing. On the morning of the test, the subject empties his bladder, and then takes 500 mg. of caffein sodium benzoate with a small amount of water. One hour later he urinates again, but this time the specimen is saved as the "control" urine. The subject now takes a 2-gm. packet of the azure-A resin, suspended in a small amount of water. Two hours later, the subject urinates again and this specimen is saved as the "assay" urine.

All the urine produced during this 2-hour interval must be included in the assay specimen, and if the specimen cannot be obtained at the end of 2 hours the examination is abandoned.

Although this test is simple enough for self-administration of the drugs and collection of urine specimens, in this entire series the medications were given and the urine samples collected at the appropriate times by a single technician. Furthermore, all the tests were conducted between the hours of 7:00 and 9:00 a.m.

The laboratory treatment of the urine samples has been well described (*35*) and will not be considered here except to emphasize its simplicity and inexpensiveness. It has been estimated that a competent laboratory technician should perform the test in 1 to 4 minutes (*27*). Furthermore, the laboratory equipment necessary for the test is remarkably uncomplicated and inexpensive, consisting entirely of collecting bottles, test tubes, hydrochloric acid, hydrion paper, a water bath, and a visual comparator block. The test result is reported as: 1) free hydrochloric acid present, 2) indeterminate, or 3) no free hydrochloric acid present. That free HCl is present is established when the urine sample is determined, by visual comparison with a standard, to contain more than 0.6 mg. of azure-A per 300 ml. of urine. No free HCl is reported when the assay sample is determined to contain less than 0.3 mg. of azure-A per 300 ml. of urine. When the concentration of the dye falls between 0.3 and 0.6 mg. per 300 ml. of urine, the test is considered indeterminate.

Urine samples may be collected on one day and not analyzed until the following day. However, we have found that in many instances there is a significant deterioration of dye intensity after about 3 days. This deterioration is minimized by acidification to *p*H 1–2 and refrigeration. Because it takes some time for all of the dye substance to be excreted from the body, an adequate interval must elapse between each test. In this study the minimal interval between one test and another was 7 days, which in our experience has been found to be more than adequate.

Results

The tubeless gastric analysis was performed one or more times on 717 Japanese subjects. Two of these subjects had had gastric surgery before administration of the test and therefore these data are excluded from present consideration. Of the remaining 715 Japanese subjects, 344 were born in the United States, and the countries of birth of the remaining 371 were as follows: Japan, 297; Hawaii, 65; Okinawa, 7; Formosa, 1;

China, 1. Although, strictly speaking, the term *isei* refers to people of Japanese origin residing in this country who were born in Japan or Okinawa, we shall, for the purposes of this study, also consider those born in Hawaii, Formosa, and China as *isei*, since it is presumed that the cultural pattern of a Japanese subject born in any of these countries has more in common with one born in Japan than one born in the United States. Thus the *isei* group numbers 371 subjects. The term *sansei* refers to people of Japanese origin who were born in this country and whose parents were both born in this country. *Nisei* also refers to people of Japanese origin born in this country, but in this case one or both parents are *isei* (not born in the United States). Thus, the group of 344 subjects born in this country can be divided into *nisei* and *sansei* (first- and second-generation Americans). However, since there are only 3 *sansei* subjects, and, therefore, 341 *nisei*, the whole group of 344 for our purposes may be considered *nisei*. It is of some interest to note that among the *isei*, with 69.3 percent of subjects, it was necessary to conduct the interview and to give instructions for the test in Japanese, whereas among the *nisei* it was necessary to converse in Japanese with only 4.9 percent of subjects.

Although a fair number of subjects had one or more repeat tests, tables 1 through 4 present the data obtained from the first test only, on each of the subjects, free, insofar as is known, of any conditions that might affect the execution or findings of the test. Table 1, then, is a tabulation of the results of the first test performed on each of 363 *isei*. These data suggest the possibility that achlorhydria may be a more frequent finding in younger males, and possibly in the very old, as compared to females. A test of the significance of the difference in the distribution of achlorhydria by age for males and females in these data shows, however, that with populations of this size such a distribution could easily have occurred by chance.[7] The data for males and females have therefore been combined. The small numbers in the younger age groups lead to some irregularity of percentages with achlorhydria, but from age 40 on, the proportions with achlorhydria progressively increase with age.

Table 2 is comprised of the tubeless gastric analysis results on Japanese subjects who were born in this country. Again there was no difference found in the occurrence of achlorhydria among males and females, and they have been combined. Unfortunately, the *nisei* fall into the younger age groups just as the *isei* have a tendency to be more populous in the older age groups. Examination of the frequency of achlorhydria in the age groups that do overlap shows there is no statistically significant

[7] We are indebted to Nathan Mantel, of the National Cancer Institute, for the method, soon to be published, used for this and subsequent tests of significance of the data presented. The data to be compared were grouped by 10-year age groups (beginning with age group 20 to 29 years) in a series of 2×2 tables with achlorhydria and no achlorhydria as one variable and sex, race, or some other designation as the other variable. The marginal totals of each age group's 2×2 table are designated M_{1i}, M_{2i}, N_{1i}, and N_{2i}, and $M_{1i} + M_{2i} = N_{1i} + N_{2i} = T_i$. The expected number is calculated for 1 cell for each 2×2 table $[E(A_i)]$ by the formula $\frac{M_{1i}N_{1i}}{T_i}$. The variance for the discrepancy between $E(A_{1i})$ and A_{1i} is given by the formula $\frac{M_{1i}M_{2i}N_{1i}N_{2i}}{T_i^2(T_i-1)} = V(A_i)$. The determination of chi square with 1 degree of freedom is, $\frac{(|\Sigma A_i - \Sigma E(A_i)| - 0.5)^2}{\Sigma V(A_i)}$.

TABLE 1.—*Tubeless gastric analysis results for Japanese subjects born in Japan, Hawaii, Okinawa, Formosa, and China (Isei)*

Age group	Total number	Indeterminate		No free HCl	
		Number	Percent	Number	Percent
Males and females					
All ages	363	31	8. 5	79	21. 8
20	17	0	0. 0	2	11. 8
30	25	0	0. 0	1	4. 0
40	37	3	8. 1	4	10. 8
50	99	6	6. 1	17	17. 2
60	122	15	12. 3	29	23. 8
70+	63	7	11. 1	26	41. 3
Males					
All ages	168	13	7. 7	42	25. 0
20	8	0	0. 0	1	12. 5
30	11	0	0. 0	1	9. 1
40	19	2	10. 5	3	15. 8
50	43	2	4. 7	8	18. 6
60	45	4	8. 9	11	24. 4
70+	42	5	11. 9	18	42. 8
Females					
All ages	195	18	9. 2	37	19. 0
20	9	0	0. 0	1	11. 1
30	14	0	0. 0	0	0. 0
40	18	1	5. 6	1	5. 6
50	56	4	7. 1	9	16. 1
60	77	11	14. 3	18	23. 4
70+	21	2	9. 5	8	38. 1

difference in the frequency of achlorhydria between the two groups. The data for the *isei* and *nisei* have therefore been combined in table 3. Thus, this table comprises all the first test results on Japanese subjects, regardless of place of birth. Both the male and female data separately and the two combined demonstrate the rise in frequency of achlorhydria with age. There is also a less rapid rise in the frequency of "indeterminate" results with age.

In comparing the frequency of achlorhydria by age among males and females in both the Caucasian and Mexican controls, no statistically significant difference was found. The sexes have therefore been combined in the data given in table 4. It was originally expected that the data from Caucasian and Mexican controls, the latter being a large portion of the Los Angeles County General Hospital admissions as well as frequent among the non-Japanese admissions to the Japanese Hospital, could be combined. It had been reported that gastric-acid secretory behavior in these racial groups did not differ (*36*). However, this has not been the case so far.

TABLE 2.—*Tubeless gastric analysis results for Japanese subjects born in the United States (Nisei)*

Age group	Total number	Indeterminate		No free HCl	
		Number	Percent	Number	Percent
Males and females					
All ages	341	8	2. 3	16	4. 7
20	40	0	0. 0	0	0. 0
30	183	6	3. 3	9	4. 9
40	107	2	1. 9	5	4. 7
50	11	0	0. 0	2	18. 2
Males					
All ages	141	4	2. 8	9	6. 4
20	15	0	0. 0	0	0. 0
30	78	3	3. 8	4	5. 1
40	42	1	2. 4	5	11. 9
50	6	0	0. 0	0	0. 0
Females					
All ages	200	4	2. 0	7	3. 5
20	25	0	0. 0	0	0. 0
30	105	3	2. 8	5	4. 8
40	65	1	1. 5	0	0. 0
50	5	0	0. 0	2	40. 0

A statistical test of the distribution of achlorhydria by age among the Japanese, as compared with the Caucasian controls (tables 3 and 4), shows no difference. The expected and observed achlorhydrics in each group differ by only one case when the pooled data are used for computing expected numbers. A comparison of the frequency of achlorhydrics by age between the Japanese and Mexican groups, however, is significantly different ($P<.01$), with an excess of achlorhydria observed, as compared to expected, in each age group but the first for Mexicans. The Mexican group also exceeds the Caucasian group significantly in the frequency of achlorhydria ($.02<P<.05$).

The percentage of subjects by age in each study group found to have achlorhydria or an indeterminate test result is shown graphically in text-figure 1. It should be kept in mind that some of these percentages are based on quite small numbers, especially in the younger age groups and in the two control populations. The increase in achlorhydria with age is readily apparent. If the indeterminates are considered to be hypochlorhydrics in some degree and grouped with the achlorhydrics, the differences between the Mexican and Caucasian groups lose statistical significance, and that between Japanese and Mexican subjects loses some of its strength ($.02<P<.05$).

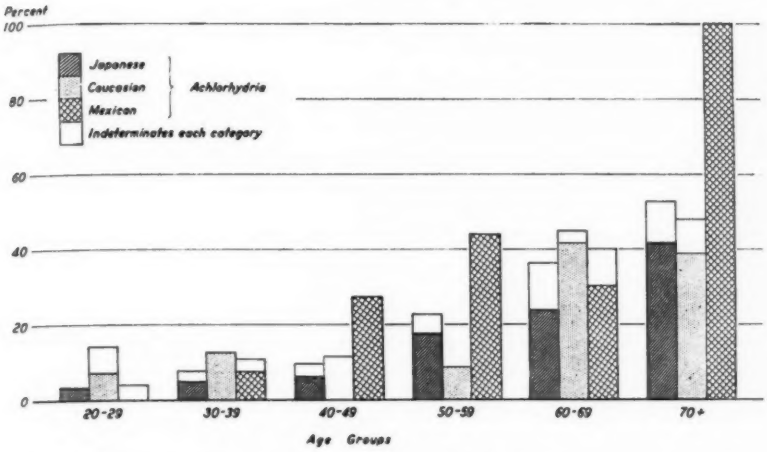
While the limited data available from the Mexican study group indicates that achlorhydria is excessive as compared to the Japanese and Caucasian

TABLE 3.—*Tubeless gastric analysis results for all Japanese subjects*

Age group	Total number	Indeterminate		No free HCl	
		Number	Percent	Number	Percent
Males and females					
All ages	704	39	5. 5	95	13. 5
20	57	0	0. 0	2	3. 5
30	208	6	2. 9	10	4. 8
40	144	5	3. 5	9	6. 2
50	110	6	5. 4	19	17. 3
60	122	15	12. 3	29	23. 8
70+	63	7	11. 1	26	41. 3
Males					
All ages	309	17	5. 5	51	16. 5
20	23	0	0. 0	1	4. 3
30	89	3	3. 4	5	5. 6
40	61	3	4. 9	8	13. 1
50	49	2	4. 1	8	16. 3
60	45	4	8. 9	11	24. 4
70+	42	5	11. 9	18	42. 8
Females					
All ages	395	22	5. 6	44	11. 1
20	34	0	0. 0	1	2. 9
30	119	3	2. 5	5	4. 2
40	83	2	2. 4	1	1. 2
50	61	4	6. 6	11	18. 0
60	77	11	14. 3	18	23. 4
70+	21	2	9. 5	8	38. 1

Table 4.—*Tubeless gastric analysis results for Caucasian and Mexican subjects*

Age group	Total number	Indeterminate		No free HCl	
		Number	Percent	Number	Percent
Caucasian					
Total	166	9	5. 4	35	21. 1
20	14	1	7. 1	1	7. 1
30	16	0	0. 0	2	12. 5
40	27	3	11. 1	0	0. 0
50	36	0	0. 0	3	8. 3
60	29	1	3. 4	12	41. 4
70+	44	4	9. 1	17	38. 6
Mexican					
Total	94	3	3. 2	18	19. 1
20	26	1	3. 8	0	0. 0
30	28	1	3. 6	2	7. 1
40	11	0	0. 0	3	27. 3
50	16	0	0. 0	7	43. 8
60	10	1	10. 0	3	30. 0
70+	3	0	0. 0	3	100. 0

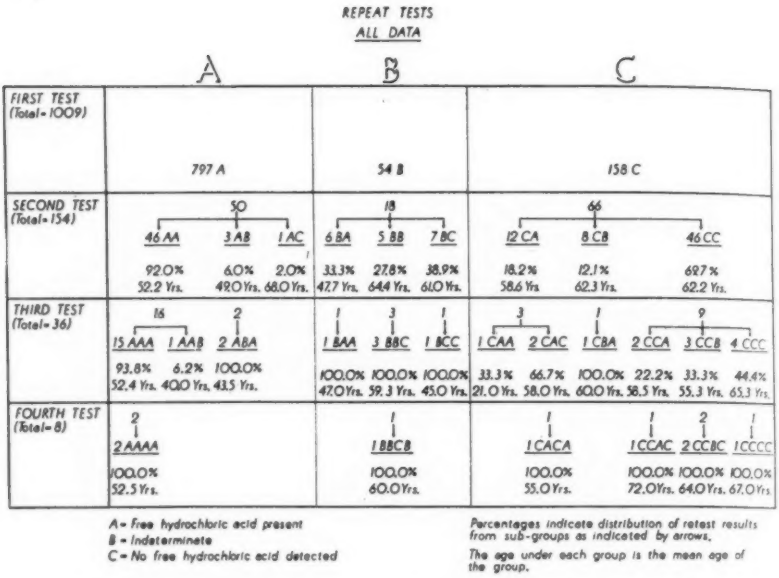


TEXT-FIGURE 1.—Percentages found achlorhydric and with indeterminate results, by age, among Japanese, Caucasian, and Mexican subjects. Based on first test by tubeless method.

groups, acceptance of this finding should await the accumulation of more data. There is no suggestion from the data obtained so far that achlorhydria is of excessive frequency among Japanese—such differences as there are, in fact, point in the other direction.

Reproducibility of Test Results Over Time for Some Subjects

The frequency of achlorhydria found here is somewhat above what other investigators have generally found through intubation gastric analysis (*18, 19, 21*). This discrepancy will be discussed and pertinent to this discussion is a consideration of the results of the tubeless method given on consecutive application to the same subject. Text-figure 2 gives the retest results among the groups having an initial test in 1 of the 3 categories of test results. (Here there are no subject exclusions since the reproducibility of the test result over time in the same subject is being considered and not the kind of test result in relation to the patient.) There were 797 subjects who demonstrated free HCl on their first test, and the test was repeated in 50 of them. Forty-six (92%) again showed free acid, with only 1 showing achlorhydria and 3 indeterminate results. The remaining repeat tests indicate that continuing to find evidence of HCl secretion is the rule, with only an occasional indeterminate reaction in this group. Eighteen of the 54 subjects with indeterminate results initially had retests that distributed them almost equally among the 3 test categories. The few additional repeat tests suggest that multiple testing would segregate these individuals as predominantly in 1 of the 3 categories, with frequent excursions into the contiguous category. From the 158 initially found not to have HCl in the stomach, two thirds of the 66 having a second test again fell in this category. Eighteen percent had


REPEAT TESTS
ALL DATA
A
B
C
FIRST TEST (Total= 1009)
797 A
54 B
158 C
SECOND TEST (Total= 154)
50
46 AA 3 AB 1 AC
92.0% 6.0% 2.0%
52.2 Yrs. 49.0 Yrs. 68.0 Yrs.
18
6 BA 5 BB 7 BC
33.3% 27.8% 38.9%
47.7 Yrs. 64.4 Yrs. 61.0 Yrs.
66
12 CA 8 CB 46 CC
18.2% 12.1% 69.7%
58.6 Yrs. 62.3 Yrs. 62.2 Yrs.
THIRD TEST (Total= 36)
16 2
15 AAA 1 AAB 2 ABA
93.8% 6.2% 100.0%
52.4 Yrs. 40.0 Yrs. 43.5 Yrs.
1 3 1
1 BAA 3 BBC 1 BCC
100.0% 100.0% 100.0%
47.0 Yrs. 59.3 Yrs. 45.0 Yrs.
3 1 9
1 CAA 2 CAC 1 CBA 2 CCA 3 CCB 4 CCC
33.3% 66.7% 100.0% 22.2% 33.3% 44.4%
21.0 Yrs. 58.0 Yrs. 60.0 Yrs. 58.5 Yrs. 55.3 Yrs. 65.3 Yrs.
FOURTH TEST (Total= 8)
2
2 AAAA
100.0%
52.5 Yrs.
1
1 BBCB
100.0%
60.0 Yrs.
1 1 2 1
1 CACA 1 CCAC 2 CCBC 1 CCCC
100.0% 100.0% 100.0% 100.0%
55.0 Yrs. 72.0 Yrs. 64.0 Yrs. 67.0 Yrs.
A = Free hydrochloric acid present
B = Indeterminate
C = No free hydrochloric acid detected
Percentages indicate distribution of retest results from sub-groups as indicated by arrows.
The age under each group is the mean age of the group.

TEXT-FIGURE 2.—Results over time from repeated tests with the tubeless gastric analysis method in the same subjects.

evidence of acid on second test. The results of further repeat testing suggests that perhaps a third of those initially having achlorhydria persist in showing this abnormality on repeated testing, with the remainder vacillating over time through all 3 categories.

Discussion

There now have been published a number of studies which establish the ability of the tubeless gastric analysis to confirm the result of the intubation (*29–34*). Fentress and Sandweiss, in their comparison of the 2 methods on 114 subjects, started the tubeless procedure immediately upon completion of the intubation examination while the stomach was still presumably under the influence of the original gastric secretory stimulant. The intubation result was confirmed by the tubeless examination in 94 percent of those who had free HCl and in 96 percent of those who lacked free HCl (*30*). Galambos and Kirsner found that with simultaneous applications of the 2 tests they were able to correctly identify 82 (tubeless) of 84 (intubation) normochlorhydric subjects and all 20 of their achlorhydric subjects (*34*). Similarly, in a more recent study, intubation and tubeless gastric analyses were performed as near to simultaneously as possible (and with the same secretory stimulant) in 114 patients who had diseases which might compromise the accuracy of the tubeless gastric method. With the exception of 7 tests performed on patients with blood urea nitrogen levels in excess of 60 mg. per 100 ml., the agreement between

the 2 methods was near perfect with only 3.5 percent of tubeless analyses showing disagreement with the intubation result as the standard reference (*33*).

In our own series, intubation was performed on 11 subjects who had had a tubeless gastric analysis and the results were qualitatively identical in all but 2. However, in this small series no attempt was made to have the 2 types of gastric analyses performed at the same time or with the same stimulant.

It would appear that if one used the same secretory stimulant and did the 2 different types of gastric analyses simultaneously, one could expect almost perfect correlation between the tube and the tubeless methods.

Gastric-acid secretory stimulants differ in their potency, and the frequency of achlorhydria within any population group will vary inversely with the potency of the gastric secretory stimulant. Poliner, Hayes, and Spiro found by the tubeless method that 20 patients were achlorhydric and when they performed intubation gastric analyses on these same patients that 15 had free acid. However, the authors point out that caffeine was used as the gastric secretory stimulant in the tubeless tests and histamine in the intubation tests, and this discrepancy may in large part be due to the difference in potency of these 2 stimulants (*37*). Vanzant *et al.* found the difference in potency between arrowroot cookies and histamine, as gastric secretory stimulants, significant enough so that they could refer to those patients who were demonstrated to have achlorhydria, with the former stimulant, as having "apparent achlorhydria," and when the latter stimulant was used, the result was considered "true achlorhydria" (*21*). In our study, caffeine was used in all tubeless tests and therefore the frequency of achlorhydria would be expected to be higher than that of a group, otherwise comparable, in which histamine was the gastric secretory stimulant used.

There is considerable evidence to support the thesis that an individual's gastric-acid secretory ability may vary greatly over periods which can be measured in hours, days, weeks, and even perhaps seasonally (*16*, *38–40*). Keefer and Bloomfield, although unimpressed with the degree to which individuals without gastric pathology (normochlorhydric) vary in their gastric-acid production (*41*), were quite impressed by the variation exhibited by subjects who as a result of one test were thought to be achlorhydric. They repeated intubation gastric analyses (Ewald's meal) on 12 patients who showed no free HCl. The interval between tests varied from a few days to a month, and the conditions under which all tests were conducted were practically identical. Seven of these subjects had no clinical evidence of disease of the stomach, and of these all but 1 were demonstrated to have acid; in 3 of them the HCl was free. In the 5 subjects with organic disease of the stomach it was not possible to demonstrate acid secretory ability on repeated examinations. Thus, in the 7 without disease the test remained negative for free HCl on subsequent examinations in only 4, or 57.2 percent (*42*). These data are in agreement with ours in that we found that if one repeated a test on a subject whose initial test

result demonstrated the presence of free HCl, the chance of confirming the original result was excellent. On the other hand, an initial result of "no free HCl detected" stood less chance of being confirmed on a subsequent test. One suspects that the group of subjects who are designated as "achlorhydric," as a result of the first test only, would be shown, if all could be repeatedly tested, to be made up of 2 types of individuals: 1) Those who are always achlorhydric (the "true achlorhydrics") and, 2) those who are rarely to almost always achlorhydric (the "intermittent achlorhydrics") under the conditions in which this particular test defines achlorhydria. Similarly, among those who are defined as normochlorhydric, as a result of the first test only, are apparently some who occasionally do not produce sufficient acid in response to caffeine to release the required amount of azure-A from the exchange resin. To follow this line of reasoning still further it would appear that those who have an "indeterminate" test result must have a *p*H of 3.2 or somewhat below, for tests *in vitro* have shown that this is the *p*H at which the release of azure-A first begins (*43*). They thus release some dye—too much to be regarded as achlorhydric, too little to be considered normochlorhydric. In subsequent tests, about one third remained in the presumed narrow *p*H range that results in an indeterminate test, another third moved to a *p*H sufficiently low to give a normochlorhydric result, and the final third to a *p*H presumably above 3.2, which results in an achlorhydric test. It would therefore be expected that if one performed multiple tests on each of the individuals in a group and one considered only those who repeatedly demonstrated achlorhydria to be the "true achlorhydrics," the frequency of achlorhydria would be less than if only one test were performed on each of the members of this group; there is an inverse relationship between the number of tests performed on each of the members of a population group and the frequency of achlorhydria of that group.

It would appear, then, that there are many factors which could influence the frequency of achlorhydria in a population group. However, the various groups in this study (tables 1 through 4) are comparable in that the same type of gastric analysis and the same gastric secretory stimulant were used in all subjects, the tests were performed at about the same hour of the day on all subjects, and only the first test results are considered.

We should expect that, if the Japanese subjects had significantly more "true achlorhydria" or "intermittent achlorhydria," this would be reflected in a higher frequency of first-test achlorhydric results in the Japanese than in the controls. Such a higher frequency does not exist and therefore it cannot be said that achlorhydria is any more frequent among Japanese than among the controls.

In clinical studies, a number which have already been cited, it has been repeatedly shown that patients with gastric cancer have about twice the frequency of achlorhydria as do persons of comparable age and sex without this disease. The general assumption has been that this association of achlorhydria and gastric cancer is indicative of some

causal relationships. It is somewhat surprising therefore to find that the threefold increased risk to gastric cancer which is characteristic of Japanese people is not reflected in the frequency of finding achlorhydria. That achlorhydria would be increased by the same factor as the gastric-cancer risk was not to be expected and, indeed, it can be shown that such an explanation would be inadequate. It seemed reasonable, however, that some increase in the frequency of achlorhydria might be found in the Japanese people.

Not all gastric-cancer patients are achlorhydric and there must be other factors than whatever may be common to the occurrence of achlorhydria and gastric cancer that influence the occurrence of the latter disease. One might speculate that it is the presence or absence of these other factors, possibly unrelated to achlorhydria, that are operating more intensely in the Japanese people and are responsible for their gastric-cancer excess.

On the other hand, the association of gastric cancer and achlorhydria may have the reverse sequential relationship to that which is usually assumed. Perhaps the occurrence of gastric cancer may frequently lead to a state of achlorhydria. One 10-year follow-up of achlorhydric patients failed to indicate that this group had an increased risk of gastric cancer because of their achlorhydric status (*44*).

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A National Cooperative Program for the Evaluation of End Results in Cancer [1]

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Summary

More than 200 hospitals in the United States are participating in a cooperative program for the evaluation of end results in cancer. Punch cards on individual cancer patients are submitted annually to the National Cancer Institute, which is sponsoring the program. A Uniform Punch Card Code has been developed which provides information on the characteristics of the patient, the primary site and extent of the tumor, the treatment given, follow-up status, and length of survival. The institutions cooperating in the End-Results Evaluation Program were selected on the basis of ability and willingness to participate. The extent to which they are representative of all hospitals treating cancer patients in the United States is not known. It is known, however, that approximately one fifth of all cancer patients in the United States are seen in the hospitals participating in the program and that a wide variety of institutions are represented, *e.g.*, cancer research centers, university hospitals, federal, State, county, and voluntary hospitals. Thus, comparisons may be made of the experience in different kinds of medical institutions. Whereas the primary goal of the program is the evaluation of end results, there will be opportunities for utilizing the resources of this group of cooperating institutions in epidemiological studies.—**J. Nat. Cancer Inst. 22: 633–646, 1959.**

History

The Cancer End-Results Evaluation Program sponsored by the National Cancer Institute of the Public Health Service is an outgrowth of the Symposium on End Results in the Treatment of Cancer at the Third National Cancer Conference, June 4 to 6, 1956 (*1*). The symposium was organized to evaluate the status of survival in cancer patients at mid-century and to examine the adequacy of available data. The data presented at the symposium indicated that only one fourth of all cancer patients, exclusive of patients with skin cancer, were alive 5 years after diagnosis. Although

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it was possible to collect information on 5-year end results on 66,000 patients from 3 central registries, 11 hospitals, and 21 individual cancer specialists, a detailed analysis could not be attempted because of the limited scope of the data collected. In addition, there was some question as to whether the data collected from the various sources were strictly comparable because of possible differences in the selection of patients, definition of terms, and follow-up policies.

At an *ad hoc* meeting of tumor registry representatives during the Conference, it was agreed that a continuing program of cooperation and coordination among the various registries was highly desirable in order to develop uniform definitions, procedures, and follow-up policies, and to pool resources for research activities. An informal organization under the name of the Association of Tumor Clinic Registrars was organized in order to promote this cooperative effort.

The recognition, by the tumor clinic registrars, of the desirability for a coordinated data-gathering program coincided with a sizable expansion of the cancer chemotherapy research program jointly sponsored by the American Cancer Society, the Damon Runyon Memorial Fund for Cancer Research, the Atomic Energy Commission, the Food and Drug Administration, the Veterans Administration, and the National Cancer Institute. The rapidly growing chemotherapy research program accentuated the need for cooperative research based on long term follow-up of cancer patients. The evaluation of chemotherapy had to be related to the end results achieved by other means of treatment. It seemed wise to build a research program for the evaluation of therapeutic end results on a cooperative effort by well-established cancer registries, and to coordinate this program with the professional societies concerned with the diagnosis, treatment, and follow-up of cancer patients. Therefore, the Cancer Chemotherapy National Service Center, the administrative unit for the national chemotherapy research program, invited the registries that had submitted usable information to the Third National Cancer Conference to join in a cooperative program for the evaluation of end results in cancer. The National Cancer Institute, of the United States Public Health Service, offered to support the research effort by contracts providing for the submission of specified data on an annual basis, and by providing a technical and administrative staff to coordinate the activities of the individual participants. Thus, the development of the chemotherapy research program offered an opportunity to achieve at least some of the hopes and aims of the tumor clinic registrars.

An organization meeting was held at the National Cancer Institute, Bethesda, Maryland, on September 12 and 13, 1956. In addition to representatives from the various tumor registries, the meeting was attended by representatives of the American Cancer Society, the American College of Radiology, the American College of Surgeons, the Association of Tumor Clinic Registrars, and the College of American Pathologists. The assembled group unanimously endorsed the proposed program and agreed that:

1) The data for the evaluation of end results would be obtained through the collection of uniform information on individual patients.
2) The participating registries would prepare and submit annually to a central repository a punch card on each cancer patient, providing information on the characteristics of the patient, the primary site and extent of the tumor, the treatment given, follow-up status, and length of survival.
3) More detailed information on selected series of cases would be collected through special studies.
4) The Cancer Chemotherapy National Service Center at the National Cancer Institute would coordinate the program and provide staff and facilities for collecting and analyzing the data.

A series of work-group meetings led to agreement on a uniform punch-card layout and code for the submission of information on individual patients. The code provided for diagnostic, treatment, and follow-up data by utilizing the information contained in the variety of code systems used by the participating registries. For the most part the data called for could be produced by mechanical procedures from the punch cards used by the individual registries. Of necessity, the uniform code was a compromise. It did not fit exactly any of the individual code systems in use, nor did it provide information in as much detail as was desirable. However, it did make possible the collection of generally comparable data from the registries participating in the program. The code was approved with the idea that experience with the use of this initial code would lead to the development of an improved uniform code, which would be generally acceptable as a mechanism for recording information within each individual registry. Thus, through the direct use of a uniform code and the application of a standard set of definitions, greater comparability of the data collected from the various registries would be assured.

During 1957 and 1958 a detailed review was made of existing procedures and definitions in each of the participating registries and of the problems involved in converting from the individual coding systems to the uniform code. This review, plus the experience of converting the information on more than 300,000 cancer patients to the initial uniform code and the experience of processing the collected punch cards, revealed the desirability of clarifying and revising a number of items. It also became evident that, in order to meet analytical goals, more detailed information was needed on treatment and a few other items.

The revised Uniform Code, effective January 1, 1959, like its predecessor, is the result of a consensus among the registries participating in the program. The code attempts to strike a balance between two essentially conflicting goals:

1) The collection of sufficient detail to permit meaningful evaluation of the efficacy of therapy.

2) The restriction of the reporting system to items of information generally available in hospital records and sufficiently simple to make possible uniform interpretation. More detailed information than provided

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tor in the code will be collected through special studies on selected groups of patients.

Since medical records are often complex, a manual of definitions, examples, and other explanatory materials is being prepared. In addition, a quality control program is being developed to check on the accuracy with which established definitions and procedures are being followed.

The manual is intended for the specific use of the participants in this particular research program, and not as a general standard. However, it is hoped that much of the material will be of use to other groups interested in the registration of cancer cases and the evaluation of end results.

Basic Philosophy

The institutions cooperating in the End-Results Evaluation Program were selected on the basis of ability and willingness to participate. The extent to which they are representative of all hospitals treating cancer patients in the United States is not known. It is known, however, that approximately one fifth of all cancer patients in the United States are seen in the hospitals participating in the program and that a wide variety of institutions are represented; *e.g.*, cancer research centers, university hospitals, and federal, State, county, and voluntary hospitals.[5] Thus, comparisons may be made of the experience in different kinds of medical institutions. It is noteworthy that the hospitals participating in the Connecticut registry program account for 94 percent of the general hospital beds in the State. All general hospitals in several metropolitan areas are in the Texas registry program. The Veterans Administration registry program will provide information on the vast majority of cancer patients seen in VA hospitals. Thus, it will be possible to compare the experience in cancer research centers and university hospitals with over-all experience in one State, several urban communities, and a national hospital system.

The annual submission of punch cards on individual cancer patients was selected as the data-collection mechanism, in preference to the submission of abstracts of case histories or the preparation of standardized statistical tabulations because:

1) The collection of standardized statistical tabulations would make for a clumsy and inflexible system. The pooling of data received from the various registries would be cumbersome and time-consuming. The tabulations called for would have to be very simple, because of the limited statistical resources of most of the registries. Whenever rearrangement of the data or tabulation of additional details was desirable, each registry would have to retabulate its own material.
2) The annual collection and processing of case-history abstracts and follow-up reports on some 50,000 new cases and some 100,000 previously reported cases would necessitate the organization of a large staff in the central office that would be duplicating a substantial part of the work performed in each individual registry.

[5] A list of participants is given in the Appendix.

3) The collection of punch cards offers several advantages—

(*a*) Punch cards provide the central office at the National Cancer Institute with a completely flexible source of information. The large volume of material being collected plus the technical and mechanical resources available to the central office make possible a large variety of comprehensive analyses previously not feasible. The availability to the central office of electronic data-processing machines makes feasible the provision of tabulating and computing services to the individual registries.

(*b*) The punch cards provide a basis for selecting samples of cases for special studies.

(*c*) Each registry is in a position to analyze its own material whenever and however it wishes.[6]

(*d*) Coding of individual records can be carried out more accurately, because the coding clerk need not rely solely on a brief record abstract. In individual hospital registries, coding problems can be resolved by referring to the patient's medical record or by seeking help from a physician familiar with the case. Central registries, such as the one in Connecticut, do not have direct access to the patient's medical record, but they can obtain additional information from individual hospitals more readily than would be possible for a national office.

(*e*) The confidentiality of information on individual patients is completely protected, because patients are identified by case number only.

Punch cards containing current follow-up information on each registered cancer case are submitted *annually* until death or "permanent loss to follow-up." This is done because experience has shown that there is no special virtue in reporting results in terms of arbitrary end points, such as 3, 5, or 10 years. The most meaningful and most reliable analyses of survival can be made by studying the survival pattern over an extended period and by utilizing all available information on all registered cases (*2*, *3*). Furthermore, this procedure provides a means for checking on the implementation of patient follow-up policies. It is generally agreed that each patient's medical condition or vital status should be determined at least once a year, either through the attending physician or by contacting the patient directly (*4*).

The participating registries are encouraged to submit punch cards on all cancer patients seen in their respective hospitals and clinics, including patients that were treated elsewhere prior to admission. Reporting of all *primary cases* (first diagnosed and/or treated in a registry hospital or clinic) is *required* of all participants in the program, and analyses of survival will usually be based on primary cases. However, several of the institutions in the program admit a substantial number of cancer patients on referral, after treatment elsewhere. Many of these patients are

[6] A duplicate set of punch cards is retained by each registry.

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candidates for treatment with endocrines, chemical agents, and other new therapeutic techniques. It is therefore important to collect information on such cases. Otherwise, no information would be routinely available on an increasingly important phase of cancer therapy. (The code provides a means for differentiating between primary and other cases.)

In accordance with the recommendations of the Joint Committee on Reporting Cancer End Results,[7] the date of initial diagnosis is used as the basic reference date. This is generally desirable, because our primary interest is the total experience of the patient, rather than an evaluation of therapy at any particular institution. Whereas the date of initial diagnosis is always used as the reference date on *primary cases*, the date of admission may be used as the reference date for cases treated prior to admission to a registry hospital. However, all such cases (*nonprimary*) must be handled in a consistent fashion by each registry. Analyses of survival from other possible reference dates, such as date of initiation of a particular course of therapy, can be carried out as special studies.

The adopted code was designed to meet the specific needs of a national program in which a large volume of information is being collected from a variety of institutions. However, the information called for is generally basic to the evaluation of cancer therapy in terms of survival. It is therefore hoped that the code and manual developed for this program may be adaptable to the needs of other end-results evaluation programs.

Discussion of Selected Items [8]

Malignancy (Field J)

The reporting of all malignant conditions is expected. This includes all neoplasms classified under rubrics 140 through 205 of the International Statistical Classification of Diseases. However, there is some disagreement concerning the malignancy of certain neoplastic conditions. For example, a papilloma of the urinary bladder may always be considered as malignant at registry A, may be classified as malignant unless specified as benign at registry B, and may be classified as benign unless specified as malignant at registry C. Thus, unless provision is made for such differences in policy, data on cancer of the urinary bladder from various registries would not be comparable. Similar problems in classification occur relatively frequently in cases with brain tumor, mixed tumor of the salivary gland, and carcinoid tumor of the gastrointestinal tract. In order to assure comparability of data for cancers of the bladder, brain, mouth, and gastrointestinal tract, all neoplasms of the 4 types mentioned above are to be reported. The code in this field distinguishes between cases that a registry had classified as malignant and would have reported anyway (code 1), and cases not classified as malignant and "reported by agreement" (code 2).

[7] The Committee was made up of representatives of the American College of Surgeons, the College of American Pathologists, the American College of Radiology, the American Cancer Society, and the U. S. Public Health Service (5).

[8] A synopsis of the code is given in the Appendix.

Stage of Disease (*Field O*)

One relatively simple classification system is being used for reporting stage of disease for all forms of cancer. Although somewhat different schemes are widely used for staging each of a number of sites, *e.g.*, breast and cervix, one system of classification is preferable in a large-scale program dealing with all forms of cancer. More detailed information on stage may be collected on a special study basis.

Clinical evaluation of the extent of neoplastic spread is frequently difficult, and the physician's opinion before treatment is unfortunately not explicitly recorded in most hospital records. It would generally be necessary for someone with medical training to review and interpret the medical record in order to obtain this information. This is not feasible on a routine basis in a large-scale registry program. Therefore, the participants in the program are asked to report information which is generally available in hospital records, namely, the evaluation of the extent of neoplastic spread at initiation of therapy, as recorded at the end of first course of treatment or first hospital discharge.

Admittedly, this is not entirely satisfactory, because discharge appraisal of stage for surgically and nonsurgically treated patients is not comparable. However, this compromise with current realities of medical records can be dealt with. Findings on differences in end results for surgical and nonsurgical therapy will be regarded as preliminary. Differences that appear to be important will be investigated through special studies, in which medical records will be abstracted by properly qualified personnel. For example, the Connecticut Department of Health and the National Cancer Institute are currently collaborating in a study to evaluate the apparently more favorable survival experience of surgically treated patients with localized cancer of the uterine cervix as contrasted with patients treated radiologically (*5*). The observed survival differential will be evaluated in the light of detailed information on the characteristics of the tumor and of the patient.

Treatment (*Fields P, Q, R*)

Provision is made for reporting the various types of treatment received by cancer patients. A distinction is made between surgery or radiation for the removal or destruction of tumor tissue and surgery or radiation for hormonal effect, *e.g.*, castration in the treatment of breast cancer. However, no attempt is being made to distinguish between radical and limited surgery, to record the amount of radiation delivered, or to specify the hormonal or chemotherapeutic agents used. Such details are best collected through special studies, as the need is recognized from the data being collected.

Information on treatment is recorded in three fields on the punch card, which, respectively, describe therapy prior to admission of the patient to the registry hospital, the first course of treatment after admission, and therapy subsequent to the first course. Major emphasis

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will be placed in evaluating end results in cases with no treatment before admission to registry hospitals.

Information on patients that were treated prior to admission to registry hospitals is being collected, because these patients constitute a substantial proportion of candidates for treatment with endocrines, chemical agents, and other new therapeutic techniques. The punch cards will provide a basis for identifying patients that were so treated. End results of therapy may then be evaluated through direct review of hospital records.

Follow-up Status and Survival (Fields T and U)

The follow-up of cancer patients is primarily oriented to the measurement of survival, which is the basic criterion for the evaluation of cancer therapy. Further information, such as the presence or absence of neoplasm at follow-up and the cause of death, is reported, when available. Follow-up techniques from one registry to another may vary, but the percent of untraced or lost cases should be kept as low as possible and in some instances has been less than 1 percent.

Survival time is being recorded, in years and months left over from date of diagnosis to date of last contact or death. This procedure permits the use of actuarial methods in analyzing survival. Thus, the *survival pattern* over an extended period can be analyzed and all cases registered as of the date of study can be included in the analysis (3). In contrast to the comprehensiveness and flexibility of the actuarial method, the practice of reporting the follow-up status as of selected anniversary dates provides rather limited information. For example, let us consider 2 hypothetical groups of 100 patients treated by method A and B, respectively. The mortality experience of the 2 groups is summarized in table 1.

TABLE 1.—*Mortality experience of 2 hypothetical groups of 100 patients*

Treatment	Number treated	Number of deaths during each year					Number alive at end of 5th year
		1st	2nd	3rd	4th	5th	
A	100	30	5	5	5	5	50
B	100	10	10	10	10	10	50

The successive annual survival rates obtained by means of the actuarial method would clearly point up the different pattern of mortality experienced by patients treated by methods A and B (*3*). In contrast, limiting the analysis to comparison of the percentage alive at the end of 5 years would lead to the erroneous conclusion that the survival results were no different for patients treated by these 2 different methods.

General Discussion

An attempt has been made to collect sufficient detail to permit meaningful evaluation of end results, without asking for more detail than is practicable in a large-scale reporting system. The information available on the punch cards will be sufficient for uncovering broad relationships. When more detailed information than provided for in the uniform code is required to resolve an issue, the punch cards may be used for selecting and identifying cases to be included in a special study.

As indicated earlier, the finding in Connecticut, that surgically treated patients with localized cancer of the uterine cervix survived longer than radiologically treated patients, is being re-evaluated through a detailed review of hospital records by a physician with statistical training. If the end results in the surgically treated patients appear to be superior, even when additional details concerning the characteristics of the tumor and the patient are taken into account, a controlled clinical trial would be called for. Thus, the relatively crude information made available by a routine reporting system served to identify an issue for more detailed investigation, which may in turn lead to the formulation of a clinical trial.

Whereas the primary goal of the program is the evaluation of end results, there will be opportunities for utilizing the resources of this group of cooperating institutions in epidemiological studies. Useful epidemiological clues have been uncovered, within individual registry systems, by a comparison of the characteristics of cancerous and noncancerous patients, or by a comparison of the characteristics of patients with one form of cancer with those of patients with other forms of cancer. For example, Lombard, in Massachusetts, has reported an association between marriage at an early age and cancer of the uterine cervix (*6*). Lilienfeld at the Roswell Park Memorial Institute found an association between smoking and cancer of the urinary bladder (*7*). By pooling the resources of the institutions participating in the End-Results Evaluation Program, it will be possible to extend this investigative technique to relatively rare cancer sites and to histological subclassifications of the more common cancer sites.

Experience to date indicates that this cooperative program is beneficial not only in that it facilitates the pooling of the resources for research activities, but also in that the exchange of ideas among the participants helps each participant clarify and improve the procedures and definitions within his local program.

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Appendix

Uniform Punch Card—Synopsis of Code

Effective January 1, 1959

A CCNSC REGISTRY NUMBER (Cols. 1–2)

B HOSPITAL IDENTIFICATION (Cols. 3–5)
- 000 Single institution registry
- 999 Class 8 case

C CASE NUMBER (Cols. 6–11)

D SEX (Col. 12)
- 1 Male
- 2 Female
- 3 Other (Hermaphrodite)
- 9 Not stated

E RACE OR COLOR (Col. 13)
- 1 White
- 2 Negro
- 3 Other
- 9 Not stated

F AGE AT DIAGNOSIS (Cols. 14–15) [special option]
In years as of last birthday
- 00 Less than 1 year
- 98 98 years or older
- 99 Unknown age

G CLASS OF CASE (Col. 16)
- 1 First diagnosed in registry hospital or clinic
- 2 Diagnosed at autopsy
- 3 Diagnosed elsewhere, no treatment prior to admission to registry
- 4 Diagnosed elsewhere, treatment initiated elsewhere and same course continued at registry institution
- 5 Diagnosed elsewhere, treated elsewhere
- 6 Diagnosed elsewhere, unknown whether treated
- 7 Consultation only
- 8 Case information obtained from death certificate only

H DATE OF INITIAL DIAGNOSIS (Cols. 17–20)
Month of diagnosis (Cols. 17–18)
- 99 Unknown month of diagnosis

Year of diagnosis (last two digits) (Cols. 19–20)
- 99 Unknown year of diagnosis
- 9999 Class 8 case or other completely unknown date of diagnosis

I DATE OF ADMISSION TO REGISTRY (Cols. 21–24)
Month of admission (Cols. 21–22)
Year of admission (Cols. 23–24)
- 0000 Not admitted to registry

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J MALIGNANCY (Col. 25)
- 1 Specified as malignant
- 2 Reportable by agreement
- 3 Followed for special interest

K PRIMARY SITE (Cols. 26–28)
Code based on International Classification of Diseases, 1955 Revision, WHO

L SEQUENCE NUMBER (Col. 29)
- 0 One primary only
- 1 First of two or more primaries
- 2 Second of two or more primaries
- 3 Third or later primary
- 9 Unspecified sequence number

M HISTOLOGICAL TYPE (Cols. 30–32)
Code per Manual of Tumor Nomenclature and Coding, American Cancer Society, 1951
- X10 Negative pathology
- X20 No histology
- X0X Pathology data not coded; not being submitted

N DIAGNOSTIC CONFIRMATION (Col. 33)
- 1 Positive histology
- 2 Positive exfoliative cytology, no information concerning histology
- 3 Positive exfoliative cytology and negative histology
- 4 Positive microscopic confirmation, method not specified
- 5 Not microscopically confirmed
- 9 Unspecified whether or not microscopically confirmed

O STAGE OF DISEASE (Col. 34) [special option]
- 0 *In situ* (only if based on histology)
- 1 Localized
- 2 Regional spread
- 3 Distant or diffuse spread
- 7 No longer localized, but unknown whether regional or distant spread
- 8 No evidence of this cancer (post-treated)
- 9 Unspecified or not recorded

P TUMOR TREATMENT—PRIOR TO ADMISSION (Cols. 35–37)
Q TUMOR TREATMENT—FIRST COURSE (Cols. 38–40)
R TUMOR TREATMENT—SUBSEQUENT COURSES (Cols. 41–43)

TUMOR TREATMENT CODE for Fields P, Q, R

Column *a* (35, 38, 41)—Surgery or Radiation to Tumor Tissue
- 1 Surgery
- 2 Radiation—Beam Therapy
- 3 (1 + 2) Surgery and Beam Radiation
- 4 Radiation—Other
- 5 (1 + 4) Surgery and Radiation—Other
- 6 (2 + 4) Beam Therapy and Other Radiation
- 7 (1 + 2 + 4) Surgery and Beam Radiation and Other Radiation
- 9 Not specified, not recorded, or information unknown
- 0 None of the above—no reason given
- X None of the above—patient refused
- Y None of the above—not recommended by physician

Column *b* (36, 39, 42)—Treatment other than Surgery or Radiation
- 1 Chemotherapy
- 2 Hormonal Treatment
- 3 (1 + 2) Chemotherapy and Hormonal Treatment
- 4 Other Cancer-Directed Therapy
- 5 (1 + 4) Chemotherapy and Other
- 6 (2 + 4) Hormonal Treatment and Other
- 7 (1 + 2 + 4) Chemotherapy, Hormonal Treatment, and Other
- 9 Not specified, not recorded, or information unknown
- 0 None of the above—no reason given
- X None of the above—patient refused
- Y None of the above—not recommended by physician

Column *c* (37, 40, 43)—Type of Hormonal Treatment
- 1 Hormones, Anti-Hormones, Steroids
- 2 Endocrine Surgery—when cancer originates at another site
- 3 (1 + 2) Hormones and Endocrine Surgery
- 4 Endocrine Radiation—when cancer originates at another site
- 5 (1 + 4) Hormones and Endocrine Radiation
- 6 (2 + 4) Endocrine Surgery and Endocrine Radiation
- 7 (1 + 2 + 4) Hormones and Endocrine Surgery and Endocrine Radiation
- 9 Incidental Surgery of an Endocrine Gland
- 0 None of the Above

S DATE OF LAST FOLLOW-UP OR DEATH (Cols. 44–47)
- Month of contact (Cols. 44–45)
- Year of contact (Cols. 46–47)

T FOLLOW-UP STATUS OR DEATH (Col. 48)
- 1 Alive at last contact—no evidence of cancer
- 2 Alive at last contact—with any cancer
- 3 Alive at last contact—cancer status unknown
- 4 Dead—no evidence of cancer at death
- 5 Dead—with any cancer
- 6 Dead—cancer status unknown

U SURVIVAL TIME SINCE DIAGNOSIS (Cols. 49–52) [special option]
- Completed years of survival (Cols. 49–50)
- Completed months "left over" (Cols. 51–52)
  - 0000 Less than 1 month survival time
  - 9999 Unknown survival time

V CAUSE OF DEATH (Cols. 53–56) International Classification of Disease, 1955 Revision, World Health Organization
- 0000 Patient alive at last contact
- 7777 No death certificate available
- 6666 Not being submitted

W AUTOPSY (Col. 57)
- 1 Yes (autopsy performed)
- 2 No (dead, no autopsy)
- 9 Unknown (dead, autopsy status unknown)
- 0 Patient alive at last contact

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YY PUNCH CARD DESCRIPTION (Col. 78)

1 First punch card for a cancer never previously reported
2 New annual punch card for a previously reported cancer
3 Correction card to replace a previously submitted punch card (patient coded as alive on previously submitted card)
4 Death correction card to replace a punch card referring to a patient previously reported as dead
5 Additional death correction card representing an additional cancer not previously reported for a patient already reported as dead

ZZ YEAR OF SUBMISSION OF PUNCH CARD DECK (Cols. 79–80)

Last two digits of year of submission

List of Participants in the End-Results Evaluation Program

Central Registries:

California Tumor Registry, Berkeley
Connecticut Cancer Case Register, Hartford
Massachusetts Cancer Register, Boston
Texas Tumor Registry, Houston
Veterans Administration, Washington

Individual Registries:

Albert Einstein Medical Center, Philadelphia
Charity Hospital of Louisiana, New Orleans
Flower-Fifth Avenue Hospitals, New York City
Indiana University Medical Center, Indianapolis
Roswell Park Memorial Institute, Buffalo
State University of Iowa Hospital, Iowa City
University of California Medical Center, San Francisco
University of Chicago Clinics, Chicago
University of Michigan Hospital, Ann Arbor
University of Virginia Hospital, Charlottesville

Electron Microscopy of Mouse Parotid Tumor (Polyoma) Virus [1]

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Summary

Crude tissue-culture fluids containing a virus which had been shown by previous workers to induce multiple tumors in mice and hamsters yielded a characteristic particle when examined with the electron microscope. Absorption and elution from red blood cells and density gradient centrifugation were utilized for further purification. The size of the particle in arrays was 40 to 45 mμ; in flattened, oblate spheroids it was 59 mμ in the major diameter and 24.2 mμ in height, which gave 44 mμ as the diameter of the sphere having the same volume as the flattened particle. Virus from 2 tissue-culture strains, one cultured from a parotid-gland tumor and the other from a mesenteric lymph-node tumor, appeared identical. Sedimentation experiments indicated that the particles were somewhat larger in solution. Particle counts from the electron micrographs were parallel to the hemagglutination (HA) titer. In one experiment the number of virus particles was 1×10^7 times the HA titer. The end point of hemagglutination corresponded to the virus dilution in which the ratio of virus particles to red blood cells was unity. The uniformity of virus size is consistent with the concept of a single virus, but morphology alone is an insufficient basis for such a conclusion.—**J. Nat. Cancer Inst. 22: 647–657, 1959.**

L. Gross (*1–4*) has described filtration, centrifugation, and electron microscopic experiments on one or more viruses obtained from extracts, the original source of which was tissue from mice with spontaneous leukemia. These extracts induced leukemia and/or parotid-gland tumors, fibromyxosarcomas, and medullary adrenal tumors following inoculation into newborn mice, of a susceptible strain. Ultrafiltration led to the conclusion that the leukemogenic property of the extracts passed through filters with a porosity of 200 mμ, while the agent inducing parotid-gland tumors passed through filters of 93 mμ. The filtration end points were not determined. The sedimentation tests suggested that the leukemogenic particles were 50 to 70 mμ in diameter.

[1] Received for publication November 4, 1958.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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The electron microscopic examination of filtrates revealed particles 30 to 75 mμ in diameter, this range superseding a previous estimate of 20 to 200 mμ (*5*). Dmochowski and Grey (*6*) found virus-like bodies in tissue sections of mice inoculated with the leukemic filtrates obtained from Gross, the over-all size ranging from 67 to 165 mμ, the cores ranging from 22 to 69 mμ.

In a recent abstract (*7*) it was stated that virus-like particles 65 mμ in size were seen in slices of parotid-gland tumor and in sections of pellets of leukemic cell extracts. These particles were similar in structure to the larger particles seen in the leukemic organs mentioned.

Stewart (*8*, *9*) also reported leukemia and diverse neoplasms from leukemic extracts of similar origin to those used by Gross. Tissue-culture methods were then introduced, with a resulting increase in tumor-inducing property but with little evidence of leukemogenic activity (*10*, *11*). Eddy, Stewart, and Berkeley (*12*) reported that gradocol membranes with a porosity of 120 mμ passed fluids causing tumors in hamsters and cytopathic changes in mouse-embryo tissue cultures. Membranes with a porosity of 77 mμ passed fluids that were ineffective.

For an electron microscopic study of these preparations, the tissue-culture material has the advantage of giving less tissue debris and also the possibility of a more simple virus population, since the leukemogenic factor has been depressed.

Materials and Methods

Virus

The source of virus for the majority of observations was supernatant fluid from 32-ounce flask cultures of mouse-embryo fibroblast tissue, representing the sixth to eighth serial tissue-culture passage of the LID-1 [3] strain of virus. Tissue cultures were prepared from trypsinized whole mouse embryo, with the use of 10 percent human serum in Eagle's basal medium for growth, and 5 percent horse serum in Eagle's basal medium for maintenance. Another strain of virus, LID-2,[4] third and fourth tissue-culture passage, was used for comparison with the LID-1 strain.

Purification and Concentration

(A) The initial procedure was to concentrate the virus by centrifugation of the tissue-culture fluid, 20,000 rpm × 2 hours in a Spinco #40 rotor after clarification at 2000 rpm × 20 minutes. The sediment was taken up in one tenth the original volume for electron microscopy.

(B) Preparations giving less cell debris were made by the following method: Partial purification and concentration of crude tissue-culture

[3] A lymphocytic neoplasm from mouse #310, passage A, (L. Gross) (*13*) was transplanted 10 times (L. Law); splenic tissue introduced into tissue culture and the tissue-culture filtrate inoculated into newborn mice resulted in a parotid-gland tumor (C. Dawe), which was introduced into mouse-embryo tissue culture (W. Rowe), and referred to as the LID-1 strain. All mice were C3Hf/Bi strain.

[4] Mesenteric lymph nodes from a C3Hf/Bi mouse with transplanted leukemia (L. Law, #12,289) from a passage A-induced leukemia (L. Gross) were put up in short-term explant tissue cultures (C. Dawe); supernatant fluids were inoculated into mouse-embryo tissue cultures (I. Brodsky), and the strain of virus recovered, designated "LID-2."

fluids was carried out by a single adsorption-elution cycle, using guinea-pig erythrocytes, followed by high-speed centrifugation. Tissue-culture fluid, clarified by centrifugation at 2000 rpm for 20 minutes, was mixed with packed guinea-pig erythrocytes to a final concentration of 5 percent and held at 4° C. for 1 hour with periodic shaking. After centrifugation at 4° C. for 15 minutes at 2000 rpm, the supernatant fluid was removed and replaced with an equal volume of phosphate-buffered saline (*p*H 7.2); the resuspended cells were placed in a 37° C. water bath for 1½ hours, with agitation at 20-minute intervals. The eluate, consisting of the supernatant fluid obtained after centrifugation at room temperature for 15 minutes at 2000 rpm, was then centrifuged in a Spinco #40 rotor at 20,000 rpm for 2 hours, and the pellet resuspended either in distilled water (1/10 volume) or in the lower one tenth of the supernatant.

For 2 preparations the order of treatment was reversed, the adsorption-elution cycle being carried out with a previously concentrated preparation. Control solutions were made by omitting the virus inoculum from the foregoing procedure.

(C) Further fractionation of the extracts was carried out by subjecting the material (B) to zonal density-gradient separation. Centrifuge tubes were partially filled with varying ratios of D_2O and H_2O, the density of the solvent varying from 1.10 at the bottom of the tubes to 1.04 at the top of the mixture. Then a zone of the virus suspension was layered on top of the density-gradient column. These tubes were placed in Spinco swinging-tube rotors. In runs with the SW-39 rotor using 5 ml. tubes, the top virus layer had a volume of 0.8 ml., while with the SW-25 rotor using 34-ml. tubes, the virus layer was 1.5 ml. in volume. After centrifuging for a time, which was found by trial to move the virus to the central region of the tubes (60 minutes at 20,000 rpm), the fluid was removed with a sampler, in 5 to 10 fractions. These fractions were then available for biological assay and electron microscopic counting of particles.

Electron Microscopy

Droplets of material (A) and its control solution were put on agar and pseudoreplicas were made of the agar surface. Materials (B) and (C) were of greater purity, and could be examined by drying droplets of the suspension on Formvar- or carbon-coated grids, without the agar pseudo-replication step.

All preparations were shadowed with chromium at an angle tan $\alpha = 2/10$ in the evaporator. The local angle of shadow on the film may differ from this by 20 percent due to tilt of the substrate.

Counting

The number of virus particles was counted on electron micrographs by a method similar to Sharp's (*14*, *15*). Nickel grids (200-mesh) were placed on a Formvar film floating on the surface of water in a large dish. Two grids and the Formvar film were then picked up with a small plastic

piate, with grids in contact with the plate and the Formvar covering the grids and plate. The plate was of a size that fitted snugly into the larger end of a standard analytical cell. After the cell was assembled, one of the various fractions of the virus-solution material (C) was introduced with a syringe through the filling hole. Since the rotor held 2 cells, 2 fractions were compared in each run.

It was found that at low speeds only a variable fraction of the virus adhered to the Formvar film on the screens. Speeds of 30,000 to 35,000 rpm were necessary for high adherence. At these speeds the particles tended to concentrate in the center of the open spaces of the grids. This indicates sagging of the Formvar with consequent movement of the particles to the outer limit of the sagging pocket. It is known from shadow experiments that the Formvar film has some sag prior to centrifugation; how much additional sag is produced in the centrifuge is unknown.

Since the particle concentration increased toward the center of the open spaces, it was necessary to count all the particles in each grid space studied rather than in random small fractional areas. For this purpose we used the lowest possible magnification in order to utilize the largest possible field for each photograph.

The hemagglutination (HA) titers were determined by the method previously described (*16*).

Results and Conclusions

Photographs of isolated particles are shown in figures 1, 2, and 3. Measurements upon such photos indicate that the diameter of the flattened, shadowed, unfixed particles is 59 ± 3 mμ. The material (A), which was a simple concentrate of the culture fluids, as well as all fractions from the density gradient runs, also gave a particle size identical to this value. Strains LID-1 and LID-2 appeared identical.

A more characteristic constant for the virus is the diameter of the desiccated particle when in the spherical form. Williams (*17*) has shown that spherical particles obtained by freeze-drying have approximately the same volume as calculated for the air-dried, flattened particles, on the assumption they have the shape of an oblate spheroid; *i.e.*, $\frac{4}{3}\pi r^3 = \frac{4}{3}\pi a^2 b$, where r is the radius of the sphere, a and b are the semimajor and minor axes of the elliptical cross section of the flattened particle. To find r, a is measured as the half width of the particle and b is determined as follows: From the equation of the tangent to the ellipse, it can be shown that

$$2b = \tan\alpha\left(X - \frac{a^2}{X}\tan\alpha\right)$$

where X is the shadow length and α the local shadowing angle. The local angle is determined by Williams' method of measuring the shadow lengths of added latex particles of known size. T. F. Anderson (*18*) showed that small particles are forced into the Formvar substrate by the surface

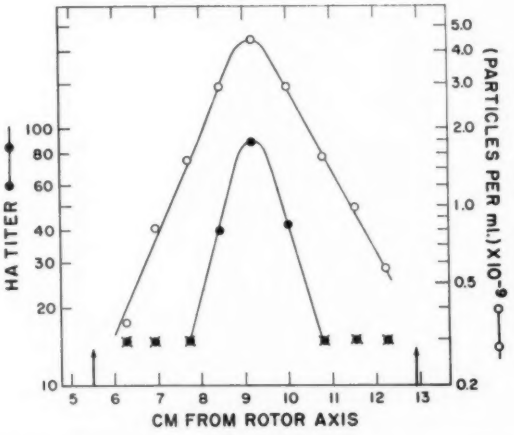
tension of the aqueous film upon drying. In our work we used both Formvar and carbon films as the substrate. Since no significant difference was seen in the shadow lengths, all values were averaged. Substituting values for X, α and a gave $2b = 24.2$ mμ and $2r = 44$ mμ for the diameter of a sphere of equivalent volume.

Measurements on groups of particles, such as those shown in figure 2, gave a value of 40 to 45 mμ for the diameter of packed particles.

These measurements indicate reasonable agreement between diameters in groups of particles and spherical diameters of isolated particles.

Five control materials consisting of supernatant fluids from tissue cultures of mouse-embryo cells, which were not inoculated with virus but treated in the same way as the inoculated fluids, gave few or no particles of the same size and appearance as these.

Text-figure 1 gives the virus counts per ml. and HA titer per 0.2 ml. for the fractions sampled from the density gradient run (material C). As would be expected, the peaks of both HA titer and number of particles are in the middle of the tube. The maximum particle counts of 4.4×10^9 per ml. correspond to the HA titer of $\frac{88}{0.2} = 440$, giving 1×10^7 particles per HA unit. Since there were 9.8×10^6 red blood cells mixed with the virus in the HA test, this gives 1 virus particle per red cell at the end point. This agrees with the findings on other viruses (*19–21*) and increases confidence that the agglutination is due to the particles counted. The recovery of virus from the fractions compared to the initial HA titer was about 90 percent.



TEXT-FIGURE 1.—Particle counts per ml. by electron microscopy for sedimented virus and hemagglutination titers per 0.2 ml. plotted against distance in rotor tube from rotor axis. The values at any distance are the averages of the two adjacent fractions. *Arrows* indicate inside and outside (top and bottom) limits of solution in tubes. Minimum dilution measurable in HA titer of the weakest fractions is 20. All values below 20 are designated by closed circles with crosses.

Infectivity assays with the adult-mouse antibody-production procedure (MAP test, *22*) were also carried out, but reliable data on sedimentation of the infectious particle relative to hemagglutinating and physical properties have not yet been obtained.

From the experiment illustrated in text-figure 1 and two more runs using HA titers, sedimentation constants of 2.87, 2.99, and 2.87 $\times$ 10^{-11} cgs units were obtained. Assuming there is a solvated density in the range of 1.20 to 1.15 leads to a diameter range of 52 mμ to 62 mμ, respectively. This range in values is due to uncertainty of measurement rather than variation in the virus.

The average 2.9 $\times$ 10^{-11} may be compared to the value 2.97 $\times$ 10^{-11} obtained for the papilloma virus (*23*).

All the data are consistent with a slightly smaller size than rabbit papilloma virus (figs. 3 and 4).

The flattening of the virus by the surface tension of the water film adhering to the substrate indicates a lack of rigidity which is shown by all animal viruses. This substantiates the hypothesis that viruses are composed of subunits (*24–26*).

The determination of the exact size of the virus in solution by the sedimentation method is important for comparison with tissue slice measurements, but this determination will require density measurements and more exact sedimentation values.

The uniformity of the virus size as seen in the electron microscope is consistent with the idea of a single virus. On the other hand, it is also possible that 2 different viruses could be so similar morphologically that they could not be differentiated by this method. It is difficult to distinguish 2 viruses that differ by 3 mμ.

The observation that similar particles are present in the crude tissue-culture fluids and in the blood-cell eluates eliminates the blood cells as the source of the particles.

The size of the virus, as reported here, falls within the range from tissue extracts reported by Gross. Since the relationship between isolated virus particles and virus as seen in tissue slices has not yet been clarified, it is uncertain how to correlate these values with the values reported by Dmochowski and Grey.

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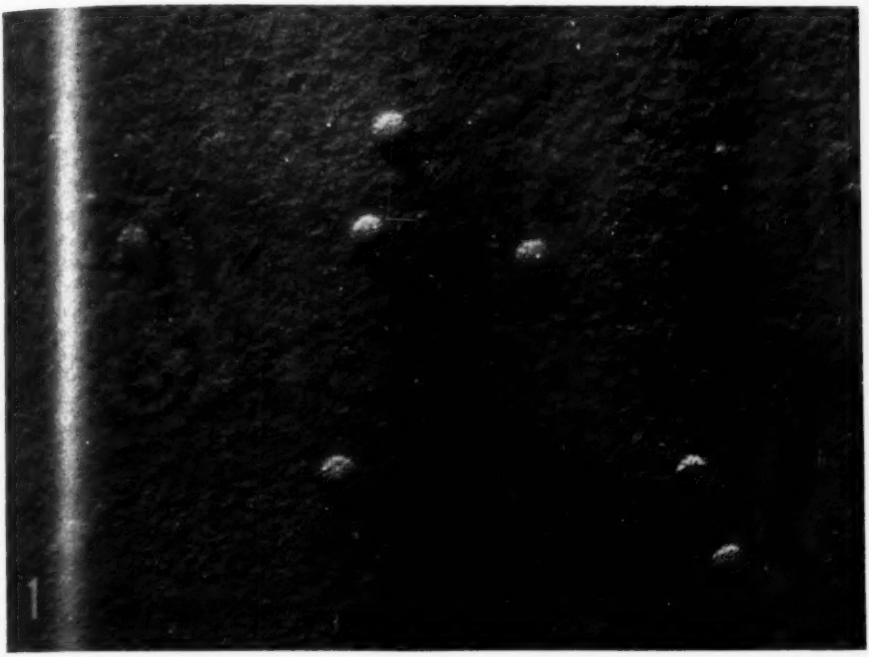
PLATE 66

FIGURE 1.—LID-1 strain of virus. All 3 figures are from eluate material, dried on Formvar and shadowed with chromium. × 98,000

FIGURE 2.—LID-1 strain of virus, slowly dried in refrigerator. × 42,700



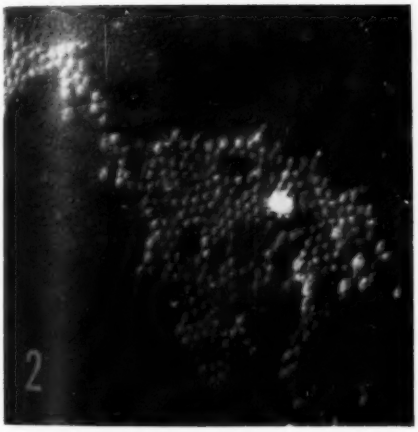


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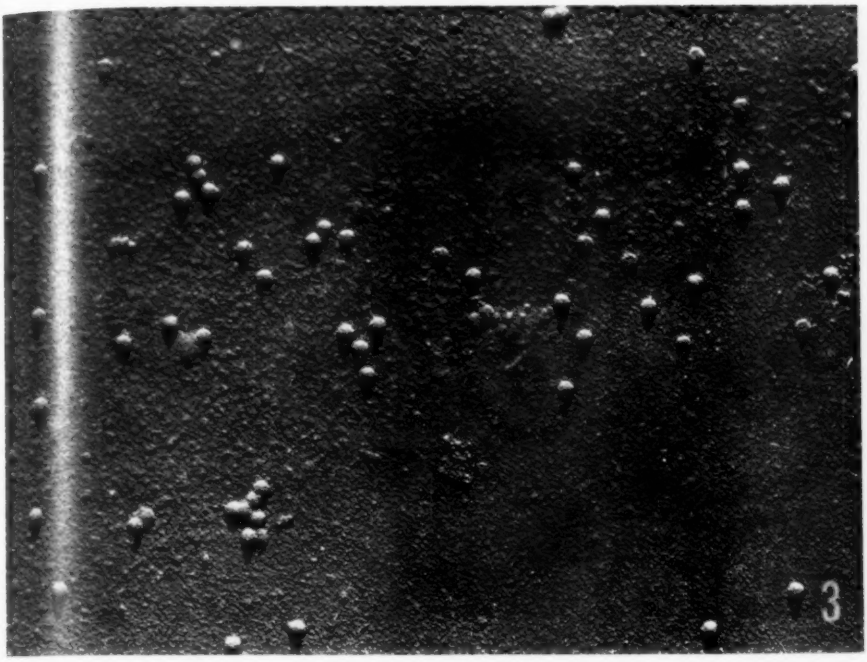
PLATE 67

FIGURE 3.—LID-1 strain of virus (*compare* with fig. 4). × 39,500

FIGURE 4.—Rabbit papilloma virus on Formvar, shadowed with chromium. × 39,500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Announcement

The Roscoe B. Jackson Memorial Laboratory, in celebration of its 30th anniversary, will hold a Symposium on June 15, 16, and 17, 1959, at Bar Harbor, Maine, on: "Some Problems of Normal and Abnormal Differentiation and Development."

Attendance will be limited to 100. Application should be made not later than April 15, to: Dr. Nathan Kaliss, Symposium Chairman, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

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